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GERMFREE VERTEBRATES: PRESENT STATUS

BY

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1-2

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Conference Chairman and Consulting Editor
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CONTENTS

Introduction

The Pure-Culture Concept and Gnotobiotics. *By* JAMES A. REYNIERS 3

Part I. Instrumentation for Rearing Germfree Vertebrates

Lightweight Stainless Steel Systems for Rearing Germfree Animals. *By* BENGT E. GUSTAFSSON 17
The Use of Plastics in the Design of Isolator Systems. *By* P. C. TREXLER 29
The Miyakawa Remote-Control Germfree Rearing Unit. *By* MASASUMI MIYAKAWA .. 37
Design and Operation of Apparatus for Rearing Germfree Animals. *By* JAMES A. REYNIERS 47
Housing of Disease-Free Vertebrates. *By* HENRY L. FOSTER 80

Part II. Rearing Germfree Vertebrates

Determination of Germfree Status. *By* MORRIS WAGNER 89
Theoretical and Practical Considerations of Germfree Animals in Virology. *By* ALTON R. TAYLOR 103
Rearing Germfree Cesarean-Born Rats, Mice, and Rabbits Through Weaning. *By* JULIAN R. PLEASANTS 116
Nutrition and Biochemistry of Germfree Chicks. *By* T. D. LUCKEY 127
Vitamin K Deficiency in Germfree Rats. *By* BENGT E. GUSTAFSSON 166
Nutrition of the Germfree Mammal. *By* BERNARD S. WOSTMANN 175
Studies on Rearing the Guinea Pig Germfree. *By* BRUCE P. PHILLIPS, PATRICIA A. WOLFE, AND HELMUT A. GORDON 183

Part III. Characteristics of Germfree and Disease-Free Animals

Morphological and Physiological Characterization of Germfree Life. *By* HELMUT A. GORDON 208
The Lymphatic System of Germfree Guinea Pigs. *By* MASASUMI MIYAKAWA 221
Some Histological and Functional Aspects of Lymphoid Tissue in Germfree Animals
  I. Morphological Studies. *By* G. J. THORBECKE 237
  II. Studies on Phagocytosis *in Vivo*. *By* G. J. THORBECKE AND B. BENACERRAF .. 247
Serum Proteins in Germfree Vertebrates. *By* BERNARD S. WOSTMANN 255
Serologic Aspects of Germfree Life. *By* MORRIS WAGNER 261

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Origin of Antihuman Blood Group B Agglutinins in Germfree Chicks. *By* GEORG F. SPRINGER, RICHARD E. HORTON, AND MARTIN FORBES 272
Disease-Free Laboratory Animals as Related to Germfree Life. *By* ROBERT D. HENTHORNE AND WAYNE O. KESTER 276

Part IV. Use of Germfree Vertebrates in Research

The Germfree Animal as a Research Tool. *By* GÖSTA GLIMSTEDT 281
A Review of Dental Research Using Germfree Animals. *By* FRANK J. ORLAND 285
A Comparison of the Development of Some Rat and Mouse Helminths in Germfree and Conventional Guinea Pigs. *By* WALTER L. NEWTON, PAUL P. WEINSTEIN, AND MYRNA F. JONES 290
The Use of Germfree Guinea Pigs in Studies on the Microbial Interrelationships in Amoebiasis. *By* BRUCE P. PHILLIPS AND PATRICIA A. WOLFE 308
Hemorrhagic Shock in Germfree Rats. *By* BENJAMIN W. ZWEIFACH 315
Role of the Intestinal Flora in the Growth Response of Chicks to Dietary Penicillin. *By* MARTIN FORBES, JAMES T. PARK, AND MEIR LEV 321
Tumorigenesis and the Germfree Chicken: a Preliminary Report. *By* JAMES A. REYNIERS AND MIRIAM R. SACKSTEDER 328
Fractionation Studies of Tumor Tissues from Germfree Chickens. *By* ALTON R. TAYLOR, FRANK B. BRANDON, AND JAMES A. REYNIERS 354

Part V. Present Status and Future Development of Germfree Life Studies

The Ecological Approach to Germfree Life Studies. *By* THEODOR JUST 371
Panel Discussion 375

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Introduction.

THE PURE-CULTURE CONCEPT AND GNOTOBIOTICS

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Introduction

The first conference on germfree life was held at the University of Notre Dame in 1939.[1] It is encouraging to note that earlier this year The New York Academy of Sciences held a conference on germfree invertebrates,[2] and that another conference was held in Sweden in connection with the Seventh International Congress for Microbiology.[3] Still other meetings are planned in the United States. All of this indicates that the study of germfree life has reached a certain maturity.

When the 1939 conference was held the only active center devoted to the study of germfree life was at the University of Notre Dame, and general scientific interest in the subject had become academic. Indeed, from 1895 to 1950, with the exception of the Lobund group, there had been but eight investigative attempts to use higher animals in such research. These studies were reported by Nuttall and Thierfelder,[4-6] Schottelius,[7-10] Cohendy and Wollman,[11, 12] Küster,[13-18] Glimstedt,[19-23] Balzam,[24] Gustafsson,[25, 26] and Miyakawa *et al.*[27-32]

Today the picture is changing rapidly. There are at present several active centers for research in this field in the United States, Sweden, and Japan.* Many more are being organized or are starting operation in this country and abroad. Interest has shifted from the academic to more applied aspects in which the animals and techniques are used to investigate problems of importance to experimental biology, chemistry, and physics. It is therefore opportune to hold this conference.

The field of gnotobiotics (*gnos*, known; *bios*, life) is new to many investigators. For this reason it may be useful to consider the subject broadly at the beginning of this monograph. The pitfalls of the past have by now been fairly well charted. Those entering the field today can purchase reliable equipment of several designs. The techniques for obtaining and rearing many germfree vertebrates have been reasonably well standardized, such animals can be shipped from production centers to the laboratory, and it is possible to rear several species through generations and in large numbers. While further development may be expected in all these areas, the position of the field is sufficiently sound so that those interested in using germfree animals can devote their efforts to the problem at hand without concern for the technical difficulties that necessarily occupied the attention of earlier investigators.

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* In the United States: Lobund Institute; National Institutes of Health, Public Health Service, Bethesda, Md.; and the Walter Reed Army Medical Center, Washington, D. C. In Sweden, the University of Lund; in Japan, Nagoya University.

A publication such as this not only serves to introduce a field to the scientific world, but can also chart the way toward a systematic development of it. At a time when there is an increasing public demand for scientific research and a great expenditure of funds, there is great need of careful planning. The widening interest in controlled environments, which covers the entire field of biology, calls for carefully considered action, since large sums of money are required and large installations are necessary. The choice before us in the United States is between an intelligent, thoughtful approach to the future, or a somewhat frantic opportunism that may generate more heat than light.

Present interest in germfree life stems from the need to standardize laboratory animals and to control their environment. This is merely an indication of the needs of biology as investigation moves from the discovery of a phenomenon to its control.[39] This need has found expression in the pure-culture concept, which is best exemplified by the development of microbiology. This concept demands that a living organism be isolated from the natural complex in which it normally exists. It may then be allowed to multiply as a "pure" animal, that is, in association only with its own kind. As such it may be allowed to live in a "controlled" environment or brought into experimental associations with other life forms. In short, experimental control rests essentially on obtaining a pure animal, or a pure culture of animals, and being able to regulate the environment involved.

The term germfree, when applied to higher animals, means that the animal so specified is free from all other life and that it exists in an uncontaminated environment. The term contaminant refers to other living beings, although it can be extended to nonliving, unwanted elements.

Such a definition may be enlarged to include living organisms that are demonstrable by existing techniques; it also may be given theoretical limits for which it would be necessary to develop new techniques and new definitions. Under any circumstance, the science or art of detecting contamination is always the limiting factor and is at best a temporary situation. However, an inquiry into possible contaminations that might be overlooked with available techniques is useful in suggesting new methods which, in turn, have significance in general biology.

Accidental contamination due to a break in isolation barriers is a technical problem, but contamination of the animal per se means that there has been a break in the surgical technique, in the maternal barriers, or a transfer through the germ cells, and is of a different order. In this area it is possible that we may be eventually forced to concern ourselves with entities that are a part of the cellular economy or close to the genetic apparatus. At any rate, the problem of detection lies within the animal.

There can never be, of course, an unequivocal answer to the question of whether an animal is germfree. For this reason I have long maintained that it is possible to say only that an animal is germfree within the limitations of the tests employed to detect contamination. These tests may very well satisfy the demands of an experiment and may not require extension. If, on the other hand, interest centers on the theoretical limits, this constitutes an investigation of considerable importance in its own right.

At this time it is possible to say that higher animals may be obtained free from contamination by bacteria, yeasts, fungi, protozoa, ectoparasites and endoparasites, and most viruses that cause a recognized disease. It is not possible to say that such an animal is or is not free from inapparent viruses except on the basis of an examination directed to that end. If something is detected that can be regarded as a contaminant, the animal is not germfree, but it may still be very useful. If the contaminant interferes with an experiment the tests for it must be included in the spectrum of tests and the search continued until animals in which the contaminant does not exist are found. It would be absurd to make an unequivocal statement that all animals are contaminated with viruses, no matter how close to the cellular economy this brings us. The important thing to remember is that an animal is germfree within the limits of the tests applied to detect contamination. The theoretical question of whether there is an absolute value to the term germfree involves a search to which there may be no end, but is nevertheless important.

Whatever definition of germfree we may adopt, it is important to consider the design of isolation barriers that make this possible, since it is within these frontiers that all work must be accomplished. In other words, when an animal is placed within a germfree system it is sealed into that system with whatever contaminants it may possess. From this point on it is necessary to be able to eradicate environmental contaminants. If this can be done successfully, then it is further possible continually to narrow the problem with respect to contaminants locked in with the animal by moving it behind new barriers as one or another contaminant is eliminated. For example, in order to press the inquiry about placental barriers, cesarotomy can be performed on germfree animals until the contaminant is eliminated or continually passes. If the contaminant is part of the genetic apparatus, the same might be said by following breeding patterns to eliminate it. The point is that the proper isolation barriers must eliminate with certainty all external contamination and so permit closer examination of internal contaminants.

A reliable apparatus makes it possible to speak of the levels of contamination in terms of the contaminants and to designate an animal as being of the first, second, third, or fourth order; the first refers to parasites, the second to microbes, the third to disease-causing viruses, and the fourth to latent or inapparent viruses.

History

It is difficult to determine the origin of the concept of germfree life, since the concepts of pure air, pure water, and pure food have been present ever since it was recognized that microbes might be involved in the health of man and animals. By the same token, the pure culture concept is buried deep in the historical development of the realization that entities must be isolated from the natural complex in which they normally exist if the demands of the experimental approach are to be satisfied.

The first direct experimentation on germfree life was done in 1885 by Duclaux,[40] who tried to raise peas and beans as pure cultures in a sterile medium.

It is interesting to speculate that in all probability Duclaux's work would have remained unknown had not Pasteur focused attention on it and on the entire problem by his remarks in his introduction to Duclaux's work.[41] Pasteur stated that he thought microbes were necessary to the life of an animal, and that life in the absence of microbes would be impossible. At any rate, it was this statement that was challenged by Nencki[42] on the grounds that microorganisms produced toxic substances, and it was in support of Nencki's veto that Nuttall and Thierfelder attempted to furnish experimental proof with respect to higher animals (guinea pigs).[4-6] This challenge was taken up by Schottelius, who tried to show that microorganisms are needed.[7-10] Again, as in the case of Pasteur and Nencki on theoretical grounds, Metchnikoff supported the thesis that microorganisms are not needed and, indeed, are harmful.[43] Cohendy[11, 44] and Cohendy and Wollman[12] supported Metchnikoff in their experiments with chickens and guinea pigs. By 1916 it was evident that chickens could live germfree much beyond the time limits set by Schottelius.

It was with Cohendy[11, 44] that the original motive for determining whether life without microorganisms would be possible began to evolve into the new concept that the germfree animal might become an experimental tool. While Cohendy did not express his observation, he was certainly not unaware of the possibility, and his observations that germfree chickens were susceptible to infection was brought out in his work with Wollman,[12] using guinea pigs infected with *Vibrio cholerae.*

Küster, working with two goats that he kept germfree for fourteen days, finally turned the tide against the theory of symbiotic helpers and opened up a wider scope to germfree experimentation.[13-18] He emphasized the importance of morphological, physiological, biochemical, and immunological examination of the germfree state and, although he did very little experimentally along these lines, his speculation is interesting.

In the period following World War I the work of Glimstedt[19-22] and Glimstedt *et al.*,[23] done at the Institute of Histology of the University of Lund, Lund, Sweden, should be especially noted. The germfree guinea pigs that he used were intended primarily as tools for investigating the lymph system. His work was followed by that of Balzam,[24] in which germfree chickens were used to investigate nutritional problems. Gustafsson, working also in the Institute of Histology at Lund, reported rearing germfree rats.[25, 26] In Japan the work of Kyoichi[45] and Miyakawa and his associates should be noted.[27-38] Because much of this work with the guinea pig is expounded in Japanese, it is not as well known as it should be.

The first long-range systematic program in the field of germfree life as it concerns higher animals was started at the University of Notre Dame in 1928 and since then has continued uninterrupted.[46] The original program was organized to investigate these five problems: (1) instrumentation, (2) methodology, (3) description of germfree life, (4) exploration of the use of the techniques and animals in problems of importance to experimental biology and medicine, and (5) the establishment of a center for germfree life studies. In realizing these objectives, the Lobund program has served well and stands as an example of the need for a continued systematic approach to studies of germfree life.

Present and Future Problems

The problem of terminology. As any field of study expands, the need for special terminology arises. The term germfree, when applied to a higher animal, has come to mean something more than is implied by the etymology of the words, and this special meaning now has the sanction of usage. However, it is not adequate to cover the broader area in which the germfree animal is but one level of ecological existence. Substitution of such terms as axenic,[47] bacteria-free,[13] pure,[41] or aseptic,[24] does not suffice.

In an attempt to state the problem, in 1949 my associates and I considered the need for a special terminology based on the pure-culture concept that underlies the conditions in which germfree life is involved.[48] One of these conditions is that an animal may be contaminated with a pure culture of microorganisms. Since the pure-culture concept supposes a knowledge of the kinds of life present in an isolated situation, we suggested the term gnotobiotics to indicate the field of investigation concerned with growing living things by themselves or in association with other known kinds of microorganisms. Using this root, the animal becomes a "gnotobiote," which can mean either that it is free from microorganisms or is in association with other known forms of life. This leaves the problem of a designation for the individuals of a pure culture that would suggest freedom from other life as "germfree" or "axenic" (*xenos*, stranger; *a*, freedom) or, more specifically, by some abbreviation or symbolism. Obviously, the basic term gnotobiotics may well suffice to describe a field of investigation, but it is not sufficient to describe the subjects of experimentation within it.

Whatever terminology is adopted, it should include the poorly defined "disease-free" or "clean" animals that originate from a germfree animal, but are conventionally contaminated except for those specific microorganisms considered undesirable. By the same token, a more satisfactory term than the conventional one is needed to describe the external environment or the normally reared animal. Finally, a proper term is needed to describe those animals in which the microbial flora is reduced or altered by antibiotics, by dietary changes, or simply by housing a conventional animal in an isolated environment.

Trends and needs in apparatus development. The prime requirement of germfree apparatus (isolators) is dependability; a second consideration is adaptability to routines. It follows that the system should be versatile with respect to experimental demands and sufficiently strong to withstand routine operations. When these demands are satisfied, the problem of cost and convenience may be considered.

Ultimately, isolation apparatus must be absolute with respect to maintaining sterility; there can be no compromise with this demand. Such rigid standards are necessarily restrictive and therefore will be reflected in the design, construction, and operation of such apparatus. Probably the closest to the ultimate design would be a completely sealed chamber, similar to a balanced aquarium, in which an ecological equilibrium could be maintained. With higher animals, however, it is necessary for practical reasons to supply food, water, and air, and to provide devices for manipulating the animals and caring for them. This means also that there must be methods for introducing animals to the isolator, of changing them from one isolator to another as they grow and multiply, and of sterilizing supplies introduced into the isolator. In short, the ultimate apparatus is a goal for design, and incorporated into it must be such things as ultimate air filters or air-sterilizing systems and flexible handling devices.

Also needed, however, is apparatus that will be less expensive initially and that can be used for short-term experimentation with reasonable certainty and at an acceptable level of control. Included here are "benchtype" designs such as hoods, jars, and envelopes that may be sterilized separately in an autoclave or by some chemical means. Such apparatus is useful to those with transient needs, especially if germfree animals can be reared in a central installation and shipped to the point of use.

In between these two extremes is apparatus simplified to the necessary degree for routine operation, but sufficiently dependable to permit long range studies and in which small colonies of germfree animals can be maintained throughout their lives. For routine operations it is important that such apparatus be standardized in construction and design.

Important in this connection is the recent development of an apparatus in which germfree animals may be transported in airplanes, railroads, or motor vehicles.[39, 49] This creates a new dimension for the use of germfree animals, since it permits the installation of one or two isolators at widely separated sites.

It seems almost certain that there will be a demand for large apparatus in which colonies of small animals may be maintained or in which larger animals such as goats, dogs, pigs, and cows can be reared. There have been several starts in this direction, but much remains to be done.[50, 51] The need for such apparatus is further emphasized in experiments in which large numbers of small animals must be used and held in the same environment. This is not easily accomplished if the animals are divided into small groups, each in its own separate isolator and environment.

One of the major problems is the further development of devices such as gloves for handling germfree animals. Inadequately made gloves once accounted for the majority of accidental contaminations, but gloves have been so improved that now they may be used successfully for as long as two years' continuous operation. Nevertheless, gloves still constitute a hazard, and further improvements are needed to strengthen them or to substitute materials other than rubber in their construction.

The dependability of steam versus chemicals for sterilization constitutes an area of investigation that can be solved effectively only by actual use over a sufficiently long period of time in operating germfree equipment. There is, however, no particular reason why the same apparatus should not be used with both steam and chemical sterilization; this is especially true, if the apparatus s constructed of stainless steel.

The cost of germfree apparatus is properly of concern to the investigator who wishes to enter the field. However, as more apparatus is produced there will be a continuing reduction in costs. At any rate, this problem must be considered in its proper perspective. Dependability and adaptability to routine operation are of primary importance. The real problem is not so much the initial cost of apparatus as the cost in time and money put into an experiment that becomes contaminated because of faulty apparatus. The present cost of dependable stainless steel equipment is about that of an autoclave and, like an autoclave, it can be expected to give many years of service. Thus the initial costs are spread over a long term. Furthermore, in the very near future it will be possible to rent or lease isolators for those with transient interest in germfree life, just as it is possible to rent hospital beds when needed.

Introduction of new species. Thus far at Lobund Institute we have reared the following species of higher animals germfree: the monkey,[52, 53] the rat,[54] the rabbit, the hamster, the mouse, the chicken,[55, 56] the turkey, and the guinea pig. Only the rat,[54] the mouse, and the chicken[57] have been bred through successive generations: the C3H mouse for 8 generations, the Swiss mouse for 11, the rat for 12, and the chicken for 2.

On the basis of information presently available, it is reasonable to believe that other species will be obtained and added to those already reared germfree. One of the neglected areas is that of working with large animals such as dogs, cats, monkeys, goats, sheep, pigs, and cows. The techniques are available and apparatus in which this can be accomplished has been produced. Another area in need of attention is that of working with cold-blooded vertebrates such as fish,[47] amphibia, and reptiles.

Hand-rearing cesarean-born mammals on sterilized diets. In the past, emphasis has been placed on obtaining weaned mammals. This was a matter of expediency. With the establishment of breeding colonies of germfree mice and rats and the weaning of rabbits, guinea pigs, and other such animals, the emphasis should change to one of studying the suckling mammal.

There is an entire area about which little is known concerning the nutritional requirements of the suckling animal, its physiology, transfer of immunity, placental protection, and biochemistry. The most direct approach to the problem is by hand feeding. Complicated as the problem seems, there is need for a systematically planned study involving different species properly selected to provide a spectrum to bear on the problem.

Thus far most attempts at rearing totally dependent suckling mammals by hand have been aimed at simulating the feeding and nursing care of the mother. In view of the difficulties inherent in this approach, especially as regards small mammals such as mice, it seems reasonable to consider nonimitative methods. Since diets must be sterilized and, by and large, approximate the composition of the mother's milk, it might be well to search in another direction. For example, instead of a highly fluid diet it might be possible to use a semisolid diet or even one containing very small pellets. The swallowing process is present in the young mammal, and devices for feeding such nutriment are available. This approach permits the use of a more highly concentrated diet with less liability of choking the young. The proportioning of ingredients required to achieve sterilization by autoclaving without clotting—a difficult thing to accomplish with concentrated liquid diets—would be eliminated.

Starting with cesarean-born young of one species, it is common practice among those interested in "clean" animals to foster-suckle the young on a lactating mother of another species. Thus, mice have been suckled on rats, rats on hamsters, and hamsters on rats and mice. The same thing can be done with germfree animals, provided the foster mother is acceptable. Foster suckling is, at best, an expedient and not a substitute for hand-rearing, and will not yield the same information.

In my laboratory partially successful attempts have been made to fix a thin-walled, small-bore tube into the stomach of the mouse for continuous and timed feeding—the so-called "pumpkin" technique. In this way automatic devices can be used to supply continuous and known amounts of liquid diet to the animal. In the same category is the self-feeding device or "artificial mother" that has been only partially successful with some species and even less so with others. These advanced techniques need more study, but they are basically workable and sound.

With larger animals such as monkeys, rabbits, dogs and, to some extent, guinea pigs, hand-feeding is less of a problem, but the physiological and nutritional factors assume greater importance. Too little is known to establish hand-feeding techniques on more than an empirical basis at the present time.

The problem of hand-feeding cesarean-born mammals should constitute an investigation in its own right, rather than be simply a means of obtaining germfree animals. Each detail of technique needs systematic study so that standards can be set up, and it is possible to discount the variables that have been inherent in past attempts.

The need for a study and description of germfree animals from birth through old age to death. The germfree animal is relatively unknown with respect to its development, anatomy, physiology, activation of defensive systems, or the reflection of the inactivation of these systems in the general economy of the animal: that is, its physiology and biochemistry and the effects of aging. The study of the germfree animal within the parameters so formed through generations and in individuals through old age to death should constitute a major effort. It is especially necessary to do this if for no other reason than that of establishing a base line relative to its use in experimental research. For example, it is especially important to know that the teeth of germfree animals develop within recognizable limits before these animals can be used in dental caries studies,[58] or to know that in radiation studies we must work with an animal whose intestinal tract is different from that of a conventional animal.[59] Moreover, a study of the germfree animal can well be considered an exploration in which new areas of research are uncovered. Finally, if the germfree animal is to find its widest use experimentally, it must be brought to certain dependable standards, and this objective cannot be achieved until a great deal more is known about the germfree animal and the state in which it lives. It is not too much to suggest that it will be necessary to study the germfree animal species by species, variety by variety, and generation by generation, lest the literature be strewn with mistakes when the technique is used experimentally and thus become discredited. The history of germfree-life studies over the past seventy years is mute testimony to the need for systematic long-range planning. During this time interest has ebbed and flowed between active efforts, and at times it has receded to a position where the animals were considered merely as laboratory curiosities.

The tendency still exists to compare the germfree directly with the conventional animal. It is natural to do this unless we consider the fact that the germfree animal has never had experience with microbial contamination, while from the moment of birth the conventional animal is host to a wide variety of contaminants over which very little experimental control can be exercised. It is true that eventually the conventional is really the state that must be investigated, and in many ways this furnishes a reason for studying germfree life. However, the conventional state can never be attained simply by contaminating an animal indiscriminately. The animal is not a test tube but a living thing that reacts to each assault upon it, and each assault leaves a mark on the complex whole of its life. Unless the tenets of the pure-culture concept are adhered to and the natural complex built up systematically in proper order, the entire value of the germfree animal can be nullified.

The problem of detecting contamination. The value of a technique lies in the number of questions it poses. In the early period of germfree life studies, interest was centered around the question of whether animals could live without bacteria. While the question was only partially answered, it furnished a stimulus for an experimental approach that has resulted in an extension of the pure-culture concept. The question of whether a germfree animal is free from viruses may very well serve the same purpose today. This is an especially opportune time to raise this question, for it can well serve to tighten up techniques beyond the practicable and toward a theoretical limit.

As matters now stand, there can be little question, that animals may be obtained free from bacteria, yeasts, fungi, protozoa, ectoparastites, and endoparasites; available techniques are adequate to demonstrate these forms of life. Moreover, the animal itself is an excellent culture medium, furnishing a variety of conditions for those forms that can be seen microscopically or as recognized disease processes. Germfree animals reared in colonies can be studied from birth, through old age to death, and through generations, so that f a contaminant manifests itself, it will not be obscured by questions of age, numbers, or generations of animals.

It is time to consider the extension of the tests presently used to detect contamination. Emphasis should be laid on the fact that new tests should be applicable to the living animal. Finding a contaminant in an animal that must be sacrificed before it can be examined will not answer the question for all animals in the group unless the contaminant can be transmitted by means other than through the genetic apparatus. As previously pointed out, once a contaminant is demonstrated the animal is obviously not germfree. It can be expected that such contaminants will be demonstrated from time to time as virus testing is incorporated into the testing routines. However, this does not mean that such a contaminant cannot be eliminated or that animals of the same species cannot be found that do not harbor the contaminant. This is a challenge for the future.

As we move from a consideration of microscopically visible contaminants to viruses that cause diseases, still another vista is opened by a consideration of those viruses ordinarily considered nonsymptomatic. This is a modern wonderland in which even the terminology is in a state of flux. Whether such viruses will manifest themselves in time as a disease in the germfree animal is a question that is now unanswered, but for which there is now some evidence. Perhaps these viruses must be brought out by forcing techniques or by passing them to another species of animal. It is possible that this path will eventually lead us to still another horizon where a new look at genetics will be required, as seems to be the case where intensive inbreeding has been practiced. The point is that such paths may be followed more certainly if the germfree animal is used. With germfree techniques and pure-culture concepts each level or step is isolated from the other.

Pathogen-free or disease-free animals. It is proper in this monograph to consider the disease-free animal, because today there is a recognized place for it. Such an animal lies in a state between that of the germfree and of the conventional animal. The increasing use of the germfree animal in experimental biology is recognized and is an indication of the trend toward standardization. Moreover, the fact that such animals are commercially available sets the pattern for wider use of them.

From the disease-free animal it is but a step toward the monocontaminated animal in which only a single kind of microbe exists. Because of a broader isolation technique the disease-free animal finds wider usage than either the monocontaminated or the germfree animal. The disease-free animal is accordingly no substitute for the closely controlled animal, which can be and is being used in studies in which such an animal is unnecessary. By the same token it can be used to investigate many problems uncovered by the study of the germfree animal.

Finally, since the disease-free animal is best obtained by contaminating cesarean-born germfree animals and must be held under controlled conditions, it is obvious that the two states have much in common.

The need for a plan to increase the availability and use of germfree animals. The problem for the future is how best to bring the germfree animal and the techniques into the laboratory so that more investigators can enter the field. Because the very nature of the technique requires specialized apparatus, certain steps should be taken to set up centers for producing germfree animals so that they may be shipped alive, or so that the products of germfree animals, such as tissues, sera, and organs may be supplied to investigators who have an interest in using them.

The key to wider use of the germfree animal lies in the fact that they may be produced at a center and shipped by public transportation or private vehicle.

This makes it possible to establish commercial centers for producing germfree animals of different species and strains. At such a center the animals may be brought to the necessary degree of genetic control and standardization. In this way small installations of one or more isolators may be located in any laboratory, since it would be unnecessary to breed animals.

The producing center can also provide isolators on a rental or lease basis. In addition, the animals may be held at the center on special diets and under conditions designated by the investigator before they are shipped for final examination. This is simply a matter of planning and coordination and can be placed on a cost basis.

Commercial centers are made possible by the advances in instrumentation, which make it possible not only to secure an operation, but also permit larger animals to be maintained. With the advance in instrumentation has come simplification and the establishment of routines. These developments have reduced the cost of germfree animals to a point close to that of disease-free animals, which are presently commercially supplied.

It is reasonable to suggest that the United States government, through its agencies, subsidize the formation of regional centers for the production of germfree animals. The objective of such centers would be limited to the production of such animals; research is best done by the individual investigator in his own laboratory. For this reason it would be best to create these centers as subsidized commercial enterprises and not to set them up within the structure of existing governmental research centers or at universities, where the stress would be on research rather than on production. Every such center, in addition to producing disease-free animals, could include a pool of germfree isolators that could be rented or leased. As suggested, such a center could provide germfree animal products for those interested in such matters as tissue culture or biochemistry, and it would have facilities for maintaining animals for given periods of time on special diets or under special environmental conditions; this would be rendered entirely feasible by the use of small isolators.

It is obvious that we are at the beginning of a new period in biology in which larger installations will be necessary. This period is marked by the biologist's awareness of the need for environmental control in the phytotrons, zootrons, and biotrons* that are presently being considered. In the past such demands for large installations have come almost exclusively from the physical sciences, for example, the atomic energy projects. With the needs presently looming on the horizon, it would be well to plan carefully so that biology may advance and realize its promise.

The present tendency toward subsidizing small individual colonies in each nstitution is probably both wasteful and unsatisfactory. Maintaining colonies of animals properly is a full-time operation involving personnel trained in veterinary medicine, genetics, microbiology, and biochemistry; consequently it involves a prohibitive cost in any but very large installations or research centers. Such centers are no substitute for research by visting scientists, who can use their time better in their own locations. The average research institution using tissue culture routinely can buy the cell lines, the media, and the containers, and thus need not maintain these lines, some of which it may use infrequently. This is also true for strains of bacteria and for culture media. There is little reason why animals should not be reared at regional centers. As matters stand at present we are years behind the needs of the times.

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* The terms "phytotron," "zootron," and "biotron" have come into use to designate apparatus or systems in which control of the entire gamut of environment of an enclosed plant or animal is possible. The term "phytotron" refers especially to plants, and involves such matters as light, temperature, and humidity. Similarly, "zootron" refers to the rearing of animals and includes, in addition, the control of noise. "Biotron," then, involves a relationship between plants and animals in a controlled environment.

Summary

Isolation techniques and apparatus for rearing and experimenting on germfree animals and for controlling the environment are available. One system (Reyniers Germfree System II), which I have described elsewhere in this monograph, has been developed and tested over a twenty-year period. The same apparatus can be used for confining dangerous pathogens in aerobiological or epidemiological experiments. While it is always possible to improve apparatus and techniques, this need no longer be emphasized. It is time to move into the study and use of germfree animals. Germfree apparatus has been standardized and is commercially available.

Some problems presently before us are concerned with terminology, improvement of apparatus, rearing new species of animals, hand-rearing cesarean-born young mammals, description of germfree animals, detection of contamination, nutrition, and the need for federal support of regional centers for producing germfree animals.

The basis of germfree life studies lies in an extension of the pure-culture concept. On this basis the term gnotobiotics has been suggested to designate the field of growing living things in pure culture or in association with other pure cultures. The field can rightly be extended to cover the presently poorly named disease-free or clean animals and toward standardization of environment and animals.

The need for germfree animals has been well demonstrated by their wide use in many different fields of research and in the growing need for better control of the environment. The intent of this monograph is to bring together information on the above-mentioned problems and to outline the present status of the field.

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Part I. Instrumentation for Rearing Germfree Vertebrates

LIGHTWEIGHT STAINLESS STEEL SYSTEMS FOR REARING GERMFREE ANIMALS*

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Introduction

During the past decade it has become apparent that the germfree rat has many possibilities in laboratory investigations. Accordingly it is important to develop apparatus and techniques that will make it possible to rear and utilize these animals for an increasing number of experiments. The present paper details some of the more pertinent changes that have been made in our earlier technique (Gustafsson, 1948).

The germfree animal must be isolated in a tank or a room in which optimal heating and ventilation are provided under germfree conditions. Any type of room or apparatus requires sterilization before use. To a large degree the mode of sterilization will be the determining factor in the design of the apparatus. One way to do this is to use steam under pressure, and to build the whole apparatus according to the principles of an autoclave, as has been done by Reyniers and his co-workers (1943, 1956). Another approach is that used by Trexler and Reynolds (1957), who sterilized a thin-walled transparent plastic apparatus by chemical means. Using the first principle, the possibility of getting complete sterilization is excellent, but the apparatus probably must be of rather heavy construction and have but a small window. In the case of the flexible plastic apparatus, problems may arise in connection with the sterilization of metal cages and instruments; moreover, the plastic is more vulnerable to cracks and breaks. There is no doubt, however, that plastic will offer many advantages when transparent plastic capable of withstanding steam sterilization becomes available. The apparatus built according to Gustafsson (1948) might be placed between the two types outlined above. It is sterilized in an autoclave with steam under pressure but, because the pressure is the same inside as outside, the thin-walled metal apparatus may be of any form and can have large glass windows.

Germfree Rearing Tanks

The larger type of germfree rearing tank intended for two operators has a volume of 700 l. and can house 20 to 30 rats (FIGURES 1, 2, and 3). Such tanks are used for breeding, for experiments in which an assistant is necessary, in studies where metabolism cages are used, and for investigations in which experimental groups of animals must be kept within the same apparatus. The other type of essentially the same construction is smaller, having a volume of 200 l., and is used for studies on as many as 10 rats. The smaller apparatus

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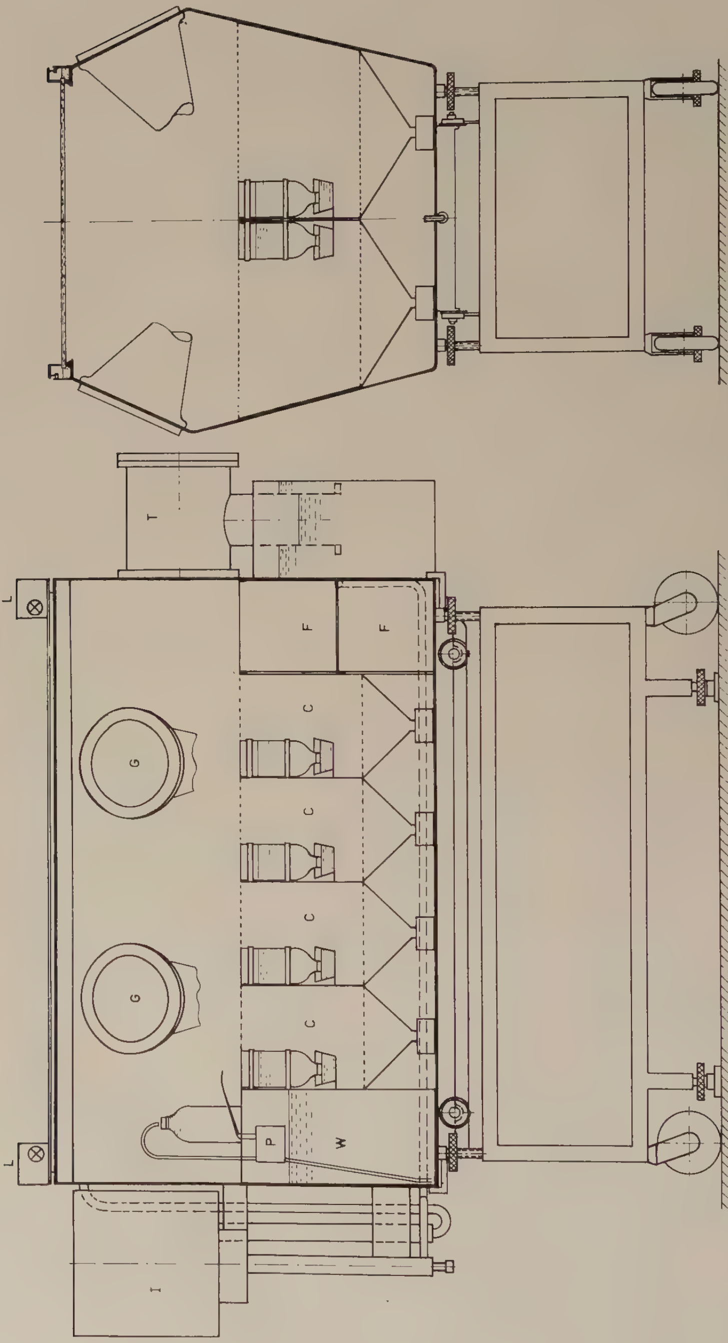

FIGURE 1. Cross-section of large germfree tank (700 l. volume). L, lighting frame; I, air sterilizer; W, water tank, with pump, P; C, cages; G, glove ports; F, food canisters; T, food autoclave and transfer unit.

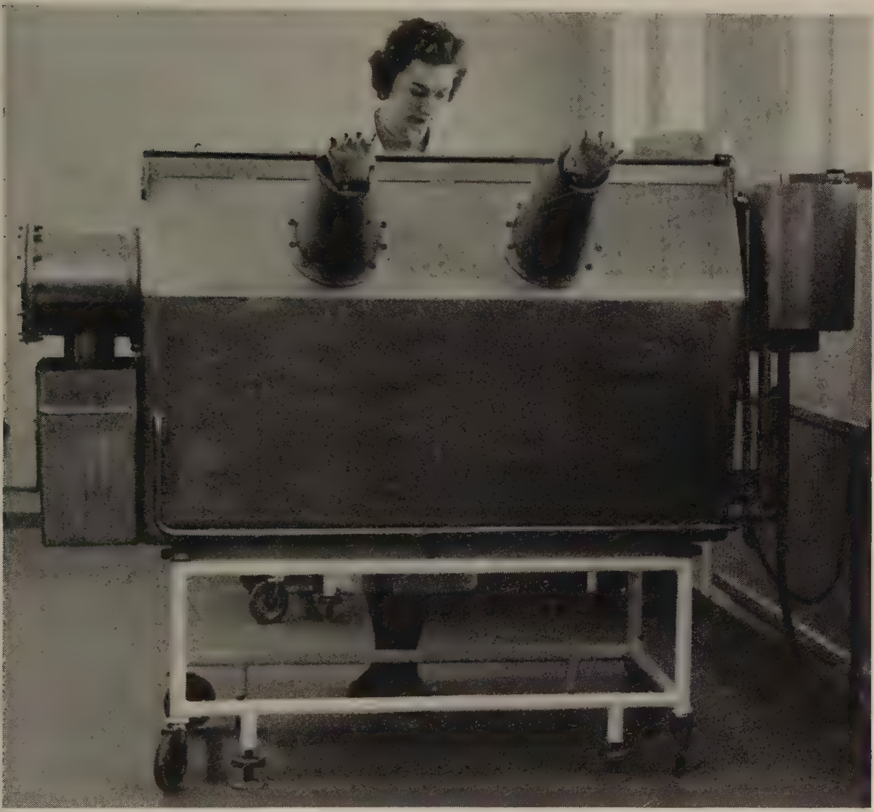

FIGURE 2. Exterior of large germfree tank (see FIGURE 1).

can easily be transported, complete with germfree animals and ready for experiments. Recently germfree animals have been transported successfully in such a standard apparatus from Sweden to the United States.

Both tanks are made of 2-mm.-thick stainless steel plate and have a square, angled design with slightly slanting sides and glove ports. The entire top is fitted with a thick glass window that also serves as the lid through which the apparatus is stocked at the beginning of an experiment. The tanks are lighted by fluorescent lamps placed above the glass window. To prevent the glass from cracking during heating it is placed on a soft rubber packing fastened by a frame in such a way that a groove is formed around the packing. After autoclaving, this groove is filled with a germicide (FIGURE 4).

Within the large apparatus there are stainless steel cages in which the animals live on raised screens over funnels used for the collection of feces and urine. Several days before expected delivery the females are put in pans with wood shavings as bedding material. By means of movable partition walls the cages can be divided into 2 to 8 compartments. The top of every cage is the working

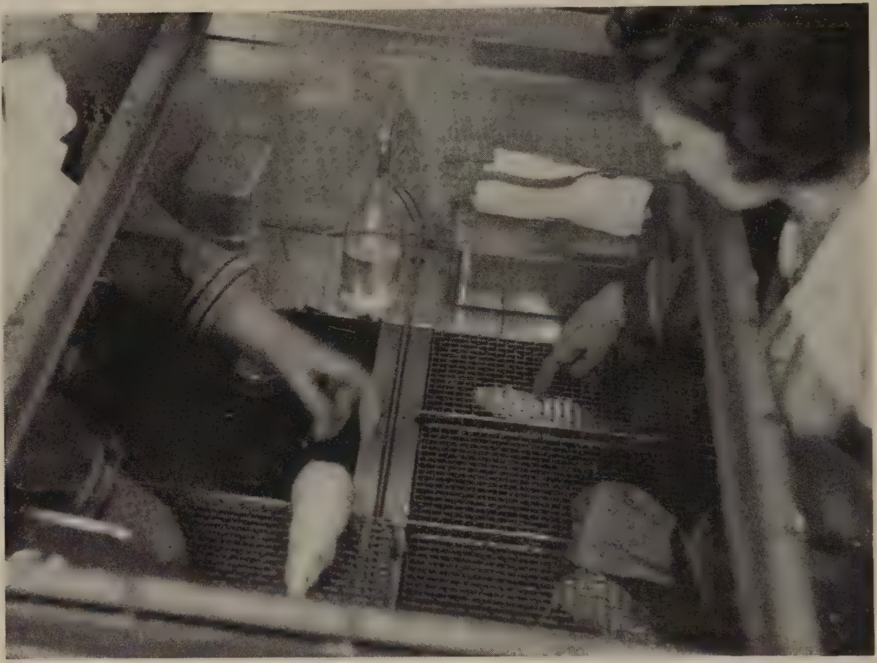

FIGURE 3. Interior of large germfree tank (see FIGURES 1 and 2).

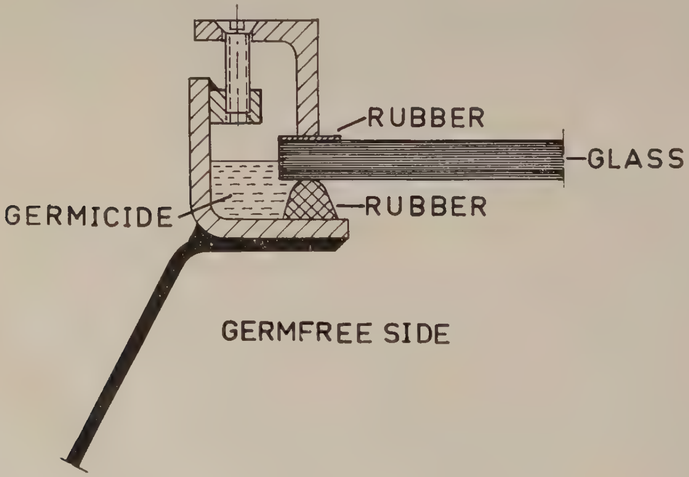

FIGURE 4. Cross-section of window packing.

surface for the operators. This surface is on the same level as the top of the 50-l. water tank and the cannisters containing 25 kg. of food (FIGURES 1 and 3). Water is pumped from the tank to fill the automatic water dispensers.

The long gloves are made in two parts. The sleeve is made of rubber, 1.5

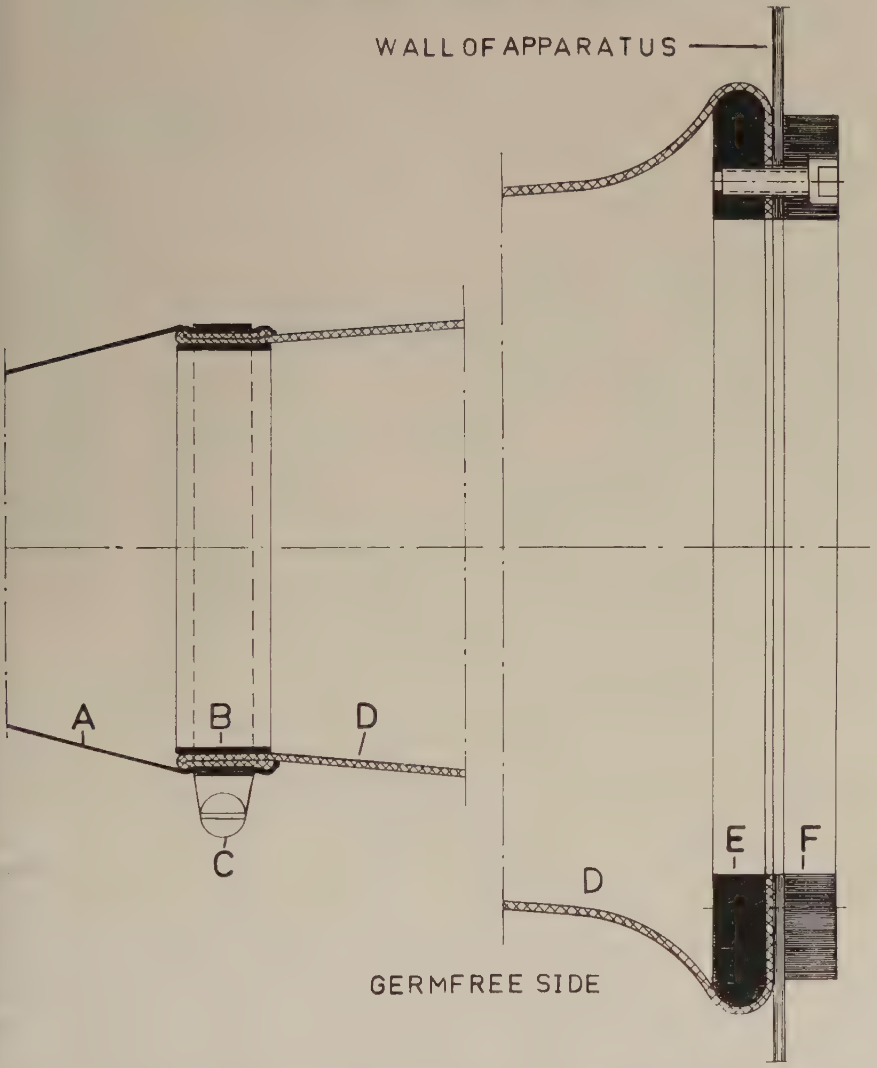

FIGURE 5. Diagram of the glove arrangement of the large germfree tank. A, cuff of ordinary glove; B, stainless steel ring; C, clamp; D, sleeve; E, inner glove ring; F, outer glove ring.

mm. in thickness, and fastened to the wall as illustrated in FIGURE 5. Heavy-duty surgical gloves are clamped on a stainless steel ring after rubber cement has been painted on the inner surface of the glove and the outer surface of the sleeve. If extra gloves are taken into the apparatus they can be changed during the experiment. The sleeves are used for several runs with an apparatus, whereas the gloves are changed before each sterilization. By this means anode-processed gloves having a high degree of thermal and physical resistance but still sufficiently thin to permit delicate operations are combined with heavy sleeves capable of withstanding considerable stretching when the operator's arms enter the apparatus.

Autoclaving of Apparatus

At the beginning of an experiment the apparatus is transferred to a vacuum autoclave, filled with all provisions for a run, and sterilized according to Gustafsson (1948), with slight modifications.

Thermocouples are placed in the food rolls and the water tank, and the cords are run out through the now empty germicide trap. An extra steam supply is run from the main inlet to the autoclave through the germicide trap to the interior of the tank. The air sterilizer is heated to 300° C. during autoclaving. After the sterilization the steam in the tank and in the autoclave is replaced with germfree air supplied through the incinerator of each apparatus.

After evacuation to 0.05 atmospheres has been established in the autoclave, the apparatus is filled with steam. The open germicide trap is now mainly a pressure leveler. When the readings of the thermocouples show that all parts of the apparatus have been kept at a temperature of 121° C. for 30 min. the steam supply is shut off and the air inlet is opened. At 0.5 atmospheres in the autoclave the germicide is pumped in through a special inlet into the germicide trap. The entire sterilization procedure takes about 3 hours, and the temperature in the food will be above 100° C. for about 1½ hours.

Air Sterilization

Air from an ordinary compressor is cooled and dehumidified by being permitted to pass through a long tube in the ground to an oil and water separator. The air is then sterilized in a combined incinerator and filter (FIGURE 6). Preheated air passes through a carborundum column heated by an electric element (350 watts) to 300° C., and then through a glass-wool filter, both being kept within the same insulated cylinder. The sterile air is then cooled by the ingoing air in a heat exchanger and by passing through a tube running along the whole length of the apparatus. Since the incinerator is never dismounted after manufacturing, the tubing from the incinerator has only welded connections. The air coming from the apparatus passes through another heat exchanger and a coil several meters long on the outside of the incinerator cylinder. The air then travels back through the heat exchanger and a flow meter that also acts as a back-check valve. In this way the outgoing air is sterilized by heat to prevent contaminated air from being sucked backward through the outlet if a negative pressure is generated in the tank. It is further obvious that the discharge into the laboratory of large amounts of contaminated air from an apparatus infected for study purposes might sometimes be a hazard. Combined incinerators and filters of the described construction have been in operation in our laboratory for several years without replenishing. Contamination of the germfree apparatus has never been traced to faulty air sterilizers.

Transfers

The apparatus is provided with sufficient food and water for a run of 2 to 3 weeks. The food is prepared by mixing with water to form a dough before

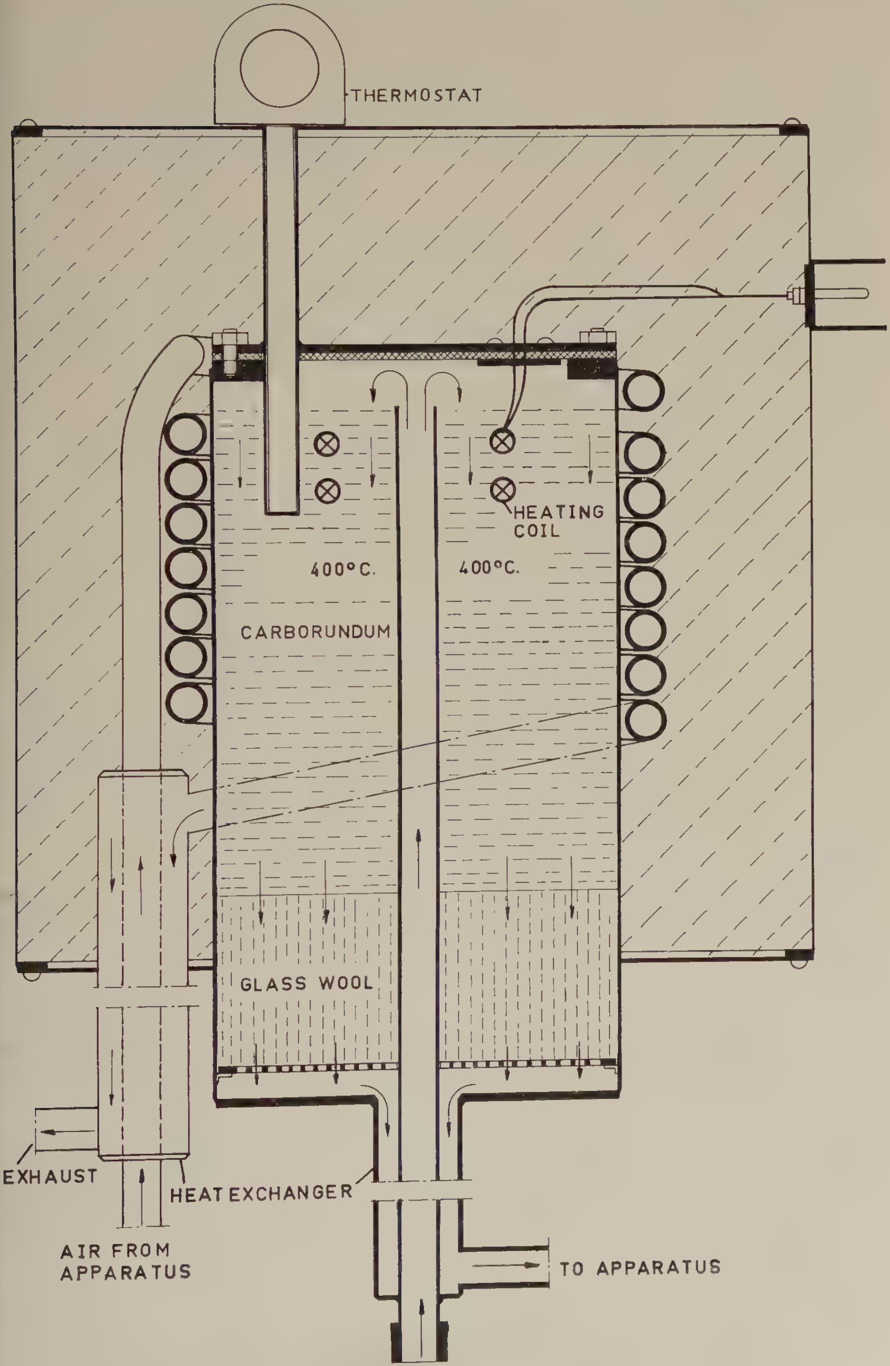

FIGURE 6. Diagram of the combined incinerator and filter for air sterilization.

TABLE 1

COMPOSITION OF DIET D7

Casein	22%
Wheat starch	63%
Arachis oil	10%
Salt mixture HMW*	4%
Vitamin mixtures	1%
Vitamins added per 100 gm. diet:	
Vitamin A	2100 I.U.
Vitamin D	450 I.U.
Vitamin E	50 mg.
Vitamin K	1 mg.
Thiamine	5 mg.
Riboflavin	2 mg.
Pyridoxine	2 mg.
Calcium pantothenate	10 mg.
Nicotinamide	20 mg.
Choline	200 mg.
Inositol	100 mg.
p-Aminobenzoic acid	30 mg.
Biotin	0.1 mg.
Folic acid	2 mg.
Vitamin B_{12}	0.002 mg.
Ascorbic acid	100 mg.

* According to Hubbell, Mendel, and Wakeman (1937).

sterilization. The composition of the stock diet is shown in TABLE 1. When the water and diet are exhausted the animals are transferred to a newly sterilized apparatus. They are transferred in glass jars sterilized with steam at the same time that the germfree tank has been sterilized. The glass jars are then brought through a combined food autoclave and germicide trap into the germicide trap of another apparatus, where they are rinsed within the apparatus in a second bath of the germicide. The objects are handled with a forceps kept in the germicide after steam sterilization. The germicide* is used in a concentration of 0.2 per cent.

Heat-labile compounds are sealed in glass ampules that are presterilized on the outside by being kept for about 2 hours in dichromate-sulfuric acid, rinsed in germicide, and taken through the trap. Glass jars with food, watercans, and so forth can also be transferred into the apparatus through the food autoclave.

None of our contaminations has been traced to a failure in the transfer procedure. It is obvious, however, that this is one of the weakest links in the long chain of operations in the presently described germfree technique. An air bubble adhering to the surface of the transferred object or any resistant organism falling into the germicide from the infected air in the laboratory might be brought in with the object. On the other hand, this technique is very convenient, as the trap need not be resterilized after use, as is the case with a food autoclave. This is of special importance in certain applied experiments in which blood samples or other materials must be taken out for immediate analysis as often as several times a day. Such a transfer is accomplished within a few seconds, and the trap is immediately ready for further use. For the transfer between tanks containing a large number of animals, the food autoclaves (FIGURES 1 and 2) can, however, be connected to each other, sterilized with steam under pressure with the inner doors and the bottom lids shut, and thus used according to the principles introduced by Reyniers and his collaborators.

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* Septin, Pharmacia, Stockholm, Sweden. This is a quaternary compound equivalent to Hyamine 1622 produced by Rohm & Haas Company, New York, N. Y.

Sterility Tests

During the experiments sterility tests are made at least once a week on feces and waste. The following culture media have been used: glucose broth, brain-liver-heart medium, thioglycollate medium and broth, Sabouraud's agar, and blood plates. Anaerobic cultures are made in a mixture of 95 per cent hydrogen and 5 per cent carbon dioxide with palladium as catalyst to remove the last traces of oxygen. Cultures are kept at room temperature and 37° C. for at least 3 weeks. Direct microscopy of fecal smears has been a valuable additional test. Recently virus tests have been begun, using tissue cultures of HeLa, amnion, and kidney cells. The animals are considered to be germfree when there is no growth on the test media. Such tests were made in our laboratory and independently, by the Department of Bacteriology of the University of Lund. The number and morphology of the few dead bacteria present in the feces are also checked. These dead bacteria can be traced to the casein used in preparing the diet. Before the construction of the present glove arrangement all experiments had ended with glove breaks. It was then found that careful watching for changes in the fecal smears was very useful, since the contaminants were sometimes difficult to cultivate.

Thus far our virus studies have shown that there are no agents in the feces that are cytopathogenic for human HeLa, amnion, and kidney cells.

Rearing Experiments

Although the apparatus may be used for any species of small laboratory animals, we have concentrated our efforts in establishing a colony of germfree rats by using animals from the Long-Evans strain reared at the Institute of Histology for more than 20 years.

Cesarean sections have been performed according to the technique described earlier (Gustafsson, 1948), and the present colony is the offspring of a litter delivered by cesarean section in June 1956. The first generation was hand fed, using an artificial milk formula of the same type as in our earlier studies, but with additions of tryptophan, lysine, and vitamin E.

The first litter of the second generation was born in September 1956, and the breeding has now proceeded to the seventh generation. As is shown in TABLE 2 the reproduction, measured as the number of young born per litter, is the same in the germfree colony as in the control colony reared in the animal room on the same sterilized diet as is used in the tanks. The control colony is of the same strain as the germfree one. When judging the rearing capacity, it must be emphasized that we have only recently had available the 5 large tanks in addition to the 6 of the smaller type. The 266 rats weaned during the 1½

TABLE 2

REPRODUCTION IN THE GERMFREE AND CONTROL COLONIES: SEPTEMBER 1956 TO FEBRUARY 1958

Number of	Germfree		Control	
		percentages*		percentages*
Litters	73		84	
Animals born	532		635	
Stillbirths	23		29	
Living young at birth	509		606	
Animals killed due to lack of space in germfree apparatus	211	42	220	36
Animals died during lactation	32	6	72	12
Animals weaned	266	52	314	52
Animals born per litter	7.3		7.6	
Animals weaned per litter	3.7		3.7	

* Percentages of living young at birth.

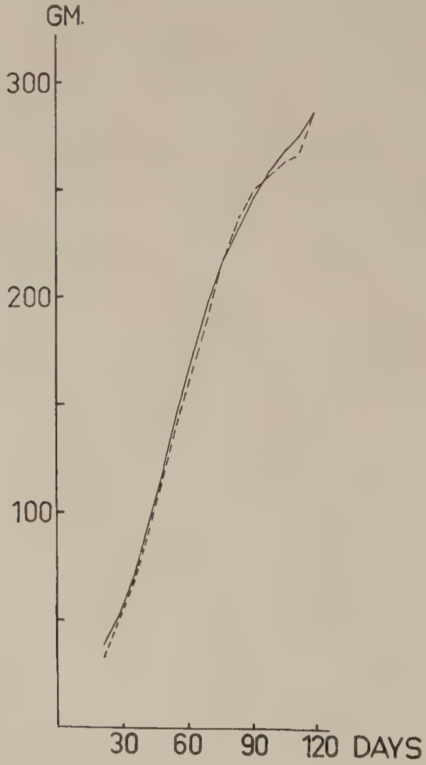

FIGURE 7. Growth curves of 18 germfree male rats (——) and 20 control male rats (- - - -) reared in the animal room on the same autoclaved diet.

years since the first litter of the second generation was born have been used for a series of published studies, including γ-globulin content of serum (Gustafsson and Laurell, 1958), the biogenesis of histamine (Gustafsson *et al.*, 1957*a*), vitamin K deficiency (Gustafsson, elsewhere in this monograph), and cholesterol and bile acid turnover (Gustafsson *et al.*, 1957*b*). Experiments in progress include the enzyme activity and bile pigments of the intestinal content, the cause of cecum enlargement of germfree animals, immunological response, total body X-ray irradiation, and morphologic examinations of tissues and organs.

As most of the animals have been used for breeding and applied experiments at the age of 60 to 70 days, only 18 males have been kept for 4 months or more. The oldest animals are at present 1½ years old. The growth curves of the 18 germfree males and 20 control males are given in FIGURE 7. The growth of both groups is good as judged by ordinary standards, and there is no difference between them. The growth of the larger number of females with an age of more than 4 months has not been charted because of the difficulties in interpreting and comparing weight curves of animals during repeated pregnancies

Contaminations

To produce the germfree colony, 41 runs with 5 small and 3 large tanks were performed prior to March 1958. TABLE 3 shows some relevant data concerning

TABLE 3

SURVEY OF COMPLETED EXPERIMENTS: SEPTEMBER 1956 TO FEBRUARY 1958

Runs of apparatus	41
Total contaminated	5
Total per cent	12
Time (days)	2304
Total per run	56
Transfers into apparatus	544
Total per run	13
Transfers out of apparatus	1260
Total per run	31

TABLE 4

SURVEY OF THE 5 CONTAMINATIONS OUT OF 41 RUNS: SEPTEMBER 1956 TO MARCH 1958

Apparatus number	Cause	Contaminants
3	Glove puncture by rat bite (rats loose in apparatus)	Animals sacrificed
6	Glove puncture by rat bite (rats loose in apparatus)	Animals sacrificed
8	Leak in food autoclave (overlooked at testing)	Mold
8	Leak in food autoclave (overlooked at testing)	Mold
8	Leak of glove sleeve (overlooked defect of manufacture)	Cocci, rods, and fungi

these runs. The sources of the contaminations could all be related to the "human factor," as presented in TABLE 4.

Summary

By the use of thin-walled stainless steel germfree tanks, rats have been reared germfree and free from demonstrable viruses in the feces up to the seventh generation. The growth and reproduction in the germfree colony were equal to those in the control animals on the same sterilized diet. The 5 contaminations in 41 runs with a total time of 2304 days were caused by glove breaks or other leaks in the wall of the apparatus. No contaminations have been traced to failures of the air sterilization incinerator or the method of transporting objects from one tank to another through germicide traps.

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THE USE OF PLASTICS IN THE DESIGN OF ISOLATOR SYSTEMS

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With few exceptions, apparatus now used for the rearing and study of the higher animals, germfree, is based upon that of Küster.[1] A sterile chamber with sight ports, attached gloves, and a sterile air supply maintains the necessary environmental conditions. Sterile objects can be removed or introduced into the chamber through a liquid germicide trap, and sterilizable objects can be moved through an attached double-doored lock or vestibule using steam under pressure. Movement of the operator is limited by the attached gloves. Such a chamber or isolator cannot accomodate large numbers of animals, the larger domestic animals, or bulky apparatus.

A large chamber or sterile room may be operated mechanically or by means of an attendant in a sterile garment. Mechanical means can be used to feed and water the animals and to move cages or lead animals into a glove-equipped chamber for examination and transfer. This requires the design of a complicated, single-purpose machine in which mechanical failure will be added to other possible hazards in this field. A sterile room serviced by attendants in garments vented to the outside requires less change of conventional practice and is a more versatile approach, since the same set-up has many uses. This course appears to be more promising at this time because a number of protective garments designed for use with chemicals, rocket fuel, radioactive contaminants, and other adverse environmental agents are available and can be modified for sterile operation.

The feasibility of the sterile room–sterile suit apparatus was determined by constructing a steam-sterilizable room[2] (8 feet in diameter by 15 feet long) called a "colony tank," in which to test entry methods and protective garments. Our experience with steam-sterilized apparatus was used in locating the source of contaminations so as to evaluate the performance of the suit. Two hundred and fifty successful entries were made with more than 1000 days of sterile operation. Twelve runs were made, all of which were eventually contaminated. The longest of these ran for 251 days. Of the 12 contaminations, one was caused by a pinhole in the garment, another was a personnel error, and the rest were due to causes connected with steel apparatus and facilities.

The feasibility of working within a sterile area was demonstrated, as were the limitations of the prototype model. Further work was discontinued because of the high contamination rate and the number of man-hours required for operation and maintenance. This result was not unexpected, since new processes usually require considerable developmental work. The successful cold sterilization of a surface as complex as that of the suit is a significant accomplishment in the development of sterile systems.

The largest single cause of contamination was glove punctures in the end tank. Gloves attached to the walls of a sterile chamber or isolator ordinarily cannot be changed without breaking sterility, while a plastic garment may be changed before every entry. The superiority of gas or spray sterilization of the garment (2 per cent peracetic acid solution) over total immersion in a germicide (3 per cent formaldehyde) was clearly demonstrated. The latter is hard on both the garment and attendant. Within the limitations of our tests the two methods do not differ significantly in ability to sterilize. The steam sterilization of a large tank obviously requires a good supply of steam and dissipates considerable heat to the surroundings.

If, as the above results indicate, the surface of a plastic garment can be spray- or fog-sterilized, then a room lined with plastic should be sterilizable by this means. A plastic-lined room would avoid the cylindrical shape, cost, and heating problems of the steam-sterilized metal tank. Preliminary studies in a 10- by 20-foot room lined with plastic indicated that this room can be sterilized by 2 per cent peracetic acid solution using either a series of permanent spray nozzles and an air-circulating fan or a portable germicide sprayer operated by an attendant in a protective garment. Resistant spores were distributed about the room in smears and on strips of filter paper. These were cultured after the room was treated. The spores were washed in neutralizing broth to eliminate bacteriostasis.

Two types of rooms have been designed and are now under construction. They will be investigated in order to determine the contamination rate and the best design for laboratory work and small-animal breeding.

Following the development of methods for sterilizing the plastic surface of protective garments, isolators patterned after Küster's original design but using flexible plastic sheeting were developed and are now under test for the rearing and study of gnotobiotes. The flexible-film isolators described by Trexler and Reynolds[3] (FIGURE 1) have been used to rear germfree mice for six months. These animals became contaminated through faulty sterilization of food and water. Square-Pak* flasks used in the preparation of sterile intravenous solution now replace cans as containers for sterile water because they can be checked readily for tightness following steam sterilization. Food is packaged in plastic bags and steam-sterilized before passage through the germicide lock. In order to avoid rupture of the package the plastic is cooled with a stream of compressed air before the pressure in the sterilizer is reduced after sterilization. The use of the Square-Pak flasks and the cooling cycle in the steam sterilizer apparently eliminates the causes of contamination in the earlier test of the plastic isolator system. Since the earlier system maintained a mouse colony sterile for 6 months, and plastic isolators with a steel end for attaching to the Reyniers' units have given little trouble, the improved system will probably function satisfactorily. It is now under test.

Isolating chambers made of flexible plastic films seem to have several advantages over those made of rigid materials. Flexible plastic films may be transparent, and their flexibility is a great aid in manipulations within the unit. Since the structure is partially sustained by the enclosed air pressure, it is impossible to produce a negative pressure, thus reducing the hazard of con-

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* Produced by the American Sterilizer Company, Erie, Pa.

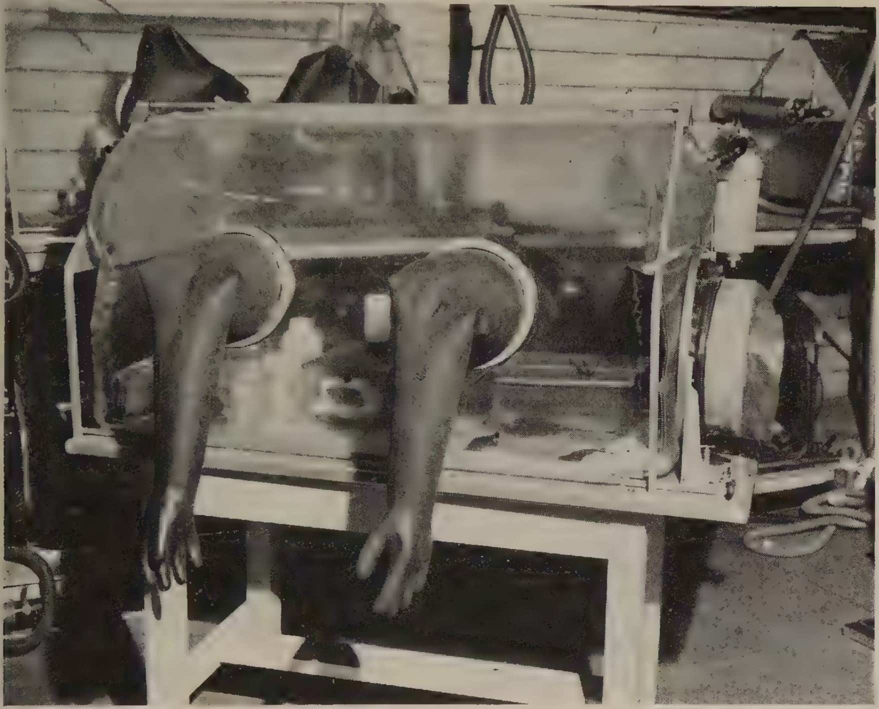

FIGURE 1. Flexible plastic isolator. Sterile lock and air outlet trap are on the right side of the isolator. The air filter is on the left and is not visible in the photograph.

tamination. The size and shape can be altered, and equipment such as wires, sensing elements, and tubing can be passed readily through the walls of the isolator. Plastic films are perhaps one of the cheapest materials that can be used in an isolator, particularly if quantities of them are produced. Eventually their cost will be reduced through design and quantity production so that isolating chambers can be discarded after use rather than be cleaned and re-sterilized.

Isolators made of plastic films are considerably lighter than those made of metal, particularly devices that must withstand sterilizing steam pressures. Because of their light weight, plastic isolators can be stacked on shelves (FIGURE 2) to conserve floor space and can be moved about readily either on stands or bench tops. Plastic isolators can be operated by technicians trained in sterile procedures. A special crew of trained men is not required, since the operation of the isolators does not involve more manipulative strength than is ordinarily demanded in a laboratory.

An obvious limitation of flexible plastic film isolators is their susceptibility to puncture. This hazard will vary with different uses, but it is significant to note that in our experience we have found that the rubber glove is more susceptible to damage than the plastic itself. The same glove is used on both

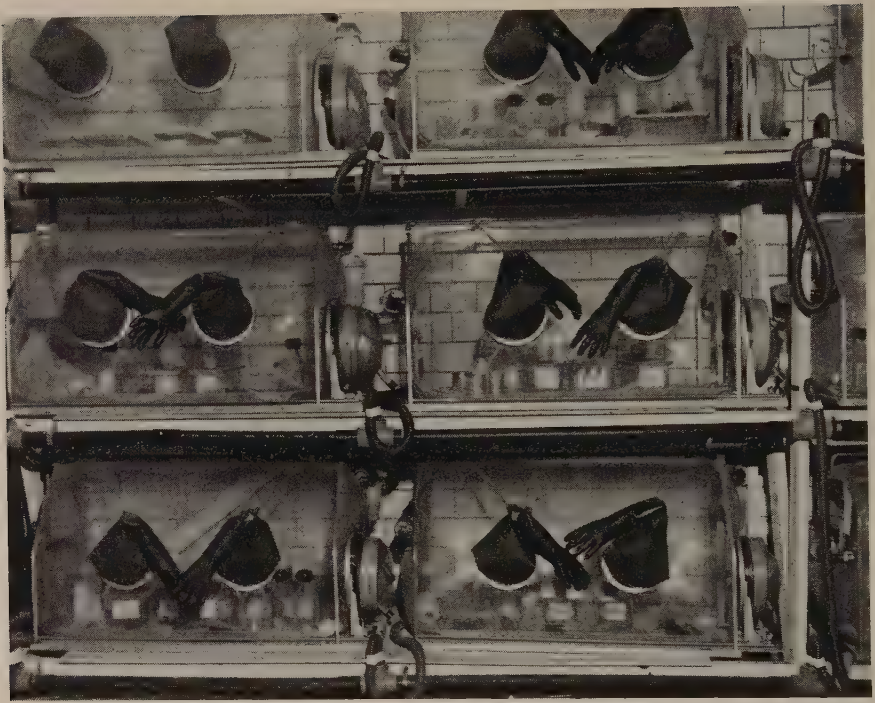

FIGURE 2. Plastic isolators stacked on shelves to conserve floor space.

plastic and metal units. Most plastics are limited to cold sterilization, although some films such as Mylar* and Trithene† can be sterilized with moist or dry heat. To date, no spores resistant to peracetic acid have been found. Another germicide, β-propiolactone, appears to be equally effective and serves as a standby should forms resistant to peracetic acid be found.

During the past year 147 gnotobiotic rats and 17 mice in 13 shipments were transported to other laboratories in plastic isolators.[4] Shipments were made in an air-conditioned station wagon equipped with a blower to supply air, and also by Railway Express. For express shipments, the isolator was placed in a protective plywood box, and a storage battery was used to operate a blower that forced air through the regular filter attached to the isolator. During the winter it was found necessary to install a thermostat to reduce the speed of the blower when the temperature of the incoming air dropped to 60° F. Only 3 shipments, involving 21 animals, were contaminated; 2 were contaminated before they left Lobund and one was contaminated en route due to an improperly attached glove. None of these failures was caused by the plastic chamber. Since these shipments were made during the course of developmental work on both the isolator and transportation methods, some failures were to be expected. Sterility is apparently maintained during shipping as securely as in the laboratory, since all of these animals were used either to establish breeding colonies or in experiments, many of them running for months.

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* Produced by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.
† Produced by Visking Corporation, Terre Haute, Ind.

Routine sterility tests at the Lobund Institute have shown that frequently contaminants are not detected for several days or weeks after an accidental break in the sterile barrier. For this reason the observations reported by Reyniers and Sacksteder[5] concerning 3 shipments of germfree animals tested for the presence of bacteria immediately upon arrival, after 24 hours in transit, have little significance as far as the determination of their germfree status is concerned. The use of a one-half-inch layer of FG-50 glass-wool mat* with a filtering efficiency of 98 to 99 per cent for bacterial and viral aerosols as reported by Decker *et al.*[6] would seem to require very careful testing in order to substantiate claims of security. The filters now used at the Lobund Institute on apparatus for the rearing and study of gnotobiotes have been challenged with a dried aerosol of washed bacterial spores at velocities encountered during use. Four layers of one-half-inch FG-50 were required to retain the viable spores even at the lowest velocities tried, less than one foot per minute. Humphrey and Gaden[7] reported an increase in efficiency of glass-wool filters with increased velocity of the bacteria-laden air stream. It would seem that the reliability of the Reyniers-Sacksteder free filters on germfree equipment in addition to transportation units, as they suggested,[5] would depend upon the absence of bacterial aerosols in the air of the laboratory.

With positive pressure maintained at all times in an isolator, as in flexible plastic film apparatus, air and specimens have been passed out directly through an opening in the wall without contamination of the interior. Entrance must be denied to flying and crawling organisms, of course. The outlet trap shown in FIGURE 3 is a variable orifice relief valve with a liquid seal that maintains a back pressure of one-fourth to three-eighths of one inch of water in the isolator. As designed, it prevents back flow, offers a free path to the escaping air, and indicates the rate of air flow.

In conclusion, it can be said reasonably that cold-sterilized flexible plastic films are suitable as sterile barriers for use in apparatus to rear, study, and transport gnotobiotes. Also, considerably more work must be done to take advantage of the unique properties of these materials and to determine the place of this apparatus in gnotobiotic technology.

Plastic films are most promising in this application because of the potential reduction of costs in the broad sense. Their transparency and flexibility reduce some of the observational and manipulative limitations of metal equipment. Rapid transfers have been made between sterile isolators by sealing together contiguous walls of plastic by heat and then cutting through the seal. Considerable reduction in the costs of components such as suits or isolating chambers can be realized with large-volume production, which may be accomplished by designing components that are useful in other fields as well.

Obviously, gnotobiotes will obtain wider use with the development of more convenient and less costly methods. At some point gnotobiotes will begin to

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* Produced by the American Air Filter Company, Louisville, Ky.

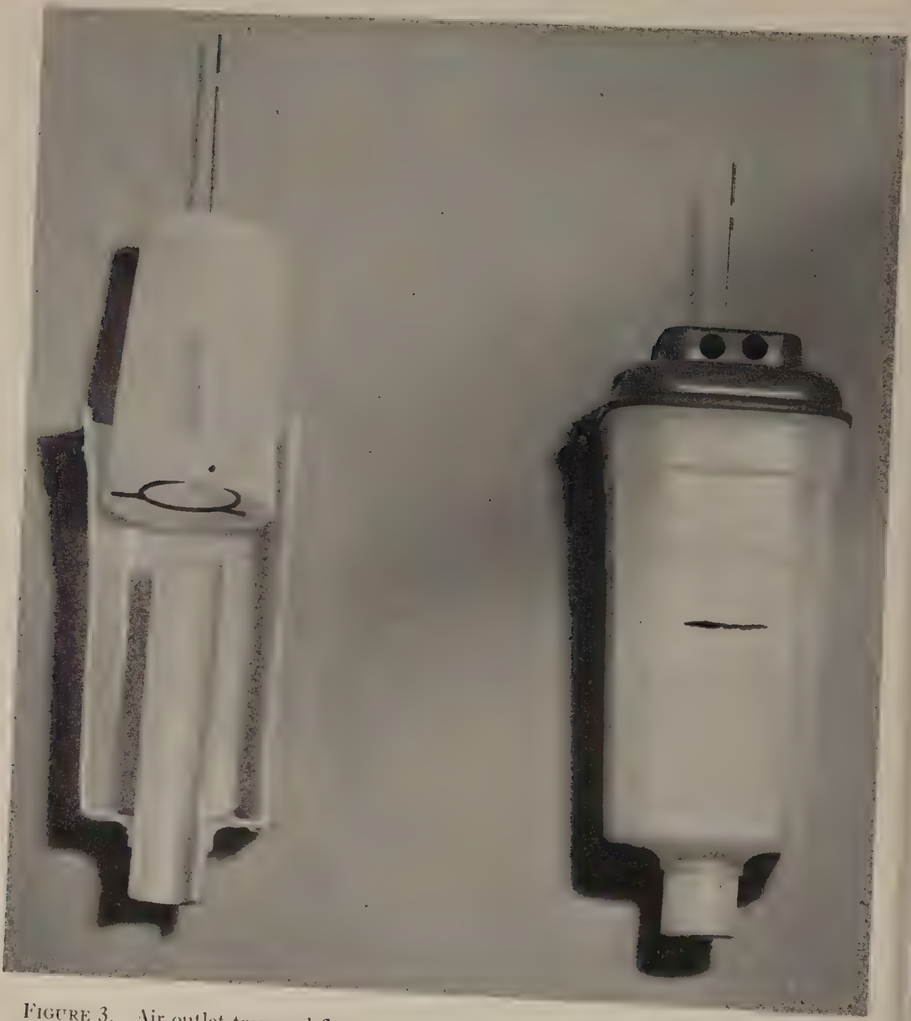

FIGURE 3. Air outlet trap and flow indicator. The section on the left shows the internal construction. Air enters through the tube (1 inch in diameter) at the bottom, elevates the float, which has been lifted above operating level in the section, and passes through the slot in the side of the float and out through the holes in the cover. The trap is filled with triaryl phosphate ester to the level mark on the outside of the container.

replace pathogen-free animals, since the gnotobiotic host can be defined more precisely. As these methods are developed, so will our knowledge of the relationship between the host and components of the associated microbiota. Conceivably, a commercial breeder will obtain what we now term germfree animals and may elect to inoculate them with one or more pure cultures rather than rear them as bacteria-free animals. These cultures will be selected because of their known effect on the host and to lessen the spread of accidental contaminants, as well as to simplify the breeder's microbiological control problem. They will also make it possible for the customer to use the animal in short-term experiments without the elaborate precautions required by germfree stock. Different associated cultures may be chosen, just as different genetic strains are now selected for various investigations. Probably a flora that resembles the important components of the human flora, either normal or diseased, can be devised to furnish gnotobiotes that are uniquely useful in toxicology or the evaluation of drugs. Such an extension of the use of gnotobiotes will be made possible by, and will contribute to, a reduction in cost of apparatus and supplies. There are definite indications that eventually food and bedding will be available in presterilized packages.

Preliminary evidence of the presence of at least 2 viruses in our so-called germfree rat colony has been obtained by Thomas G. Ward, the virologist at Lobund Institute. This demonstrates the inadequacy of the term germfree and the need for alpha gnotobiotes, as described by Trexler and Reynolds.[3] An alpha colony requires careful maintenance to eliminate exocontamination so as to preserve the gnotobiotic strain indefinitely. Periodic examination may be made in order to determine changes in the population of associated organisms and to check for new forms as they are described. These animals may be considered as biological reference points within the limits of genetic variation.

The methods presently used are quite inadequate to maintain alpha colonies, since contaminations do occur. Two methods have been proposed by Trexler and Reynolds:[3] (1) physical checks for the integrity of the sterile barrier, and (2) a germicidal barrier obtained by operating an isolator in a sterile room with a germicidal atmosphere.

In my opinion, the use of plastics will play a major role in the development of gnotobiotic technology. Fortunately one of the larger manufacturers of sterile apparatus, the American Sterilizer Company, Erie, Pa., has taken an interest in the problem. This should do much to hasten the day when an investigator can decide whether or not to use gnotobiotic animals.

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Discussion of the Paper

James A. Reyniers: As regards Trexler's observations on the use of free filters to maintain animals germfree during shipment: the use of the free filter, in the form of the universally used cotton plug, dates back to the very beginnings of bacteriology. Even apart from this, however, is our record of many shipments using the free filter without contamination. Animals can be held under 4 layers of FG-50 filter mat (the same number of layers that are and have been used on germfree isolators for several years) but, on the basis of experiment and experience, we do not think that more than 2 layers are necessary for most purposes.

Apart from this, a simple inquiry would have revealed the fact that we have held animals germfree for 10 days, which is more than ample time to demonstrate infection. Some animals were examined at the end of this period, and others were returned to the germfree isolator for extensive observation. They were free from demonstrable contamination. We are continuing to use the free filter with a simple glass jar for shipping germfree animals.

Finally, the purpose of a shipping container is to transport the animal in the shortest possible time. The real test is use, and not in theory or in attempted breakdown tests. We are satisfied that free filters are adequate for the purpose of shipping germfree animals and may very well be used on germfree isolators.

THE MIYAKAWA REMOTE-CONTROL GERMFREE REARING UNIT

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In 1946, in an attempt to rear germfree guinea pigs in a germfree rearing system, my colleagues and I constructed four different germfree rearing units of our own original design; namely, Types Nagai I, II, III, and IV. The results we obtained with these units were unsatisfactory. In 1951 we completed a closed metal germfree rearing system, the inside of which it was possible to sterilize with steam under pressure. This unit we named Type M-51 (FIGURE 1), and by 1954 this unit enabled us to extend the rearing period to a maximum of 150 days.[1] In 1956, with improved design and conception, the Type M-56 germfree rearing system was completed (FIGURE 2). In 1957, in place of the previously used rubber gloves, a set of remote-control apparatus was installed in a newly built large rearing unit, Type M-57 (FIGURE 3).

Methods and Techniques

In order to obtain germfree infants a cesarean section is performed on a term mother animal within the operating unit. On the central part of the floor of the operating unit there is an operating orifice covered with a Nylon sheet on which a square frame is provided and filled with germicide. All surgical procedure is carried on in the germicide. In order to ensure the germfree state of the young, the amniotic membrane and umbilical cords are placed in the culture media. The infants are then removed and placed in a pan, which is passed into the large-sized rearing unit. This unit, which is equipped with a set of remote-control manipulators, is a steel cylinder 1350 mm. in diameter and 2500 mm. in length (FIGURE 4).

On top of the rearing unit is an iron neck 1050 mm. in diameter and 1045 mm. in height through which the arms of the remote-control apparatus are tightly inserted and in which they form the first joints. From this point they extend down into the rearing unit, and at each end they form a second set of joints, where they become artificial fingers. The joints are covered with silicon rubber. Thus the remote-control apparatus is set tightly enough to prevent the entrance of any microorganisms from outside the unit. When the rearing unit is sterilized with steam under pressure, the outside arms of the manipulator are detached at the point where the arms enter the neck, and the outside part of the apparatus is removed. The remaining openings are covered with iron lids. In order to prevent a blowout of the rubber that covers the joints of the remote-control apparatus when the air in the unit becomes cool after sterilization by steam under pressure, sterilized air is introduced through an air pipe to balance the pressure inside the unit.

After the inside of the rearing unit has become dry and the remote-control apparatus has been reassembled to make the complete form, the pan with the young is passed into the connecting link, and the door is then sealed off

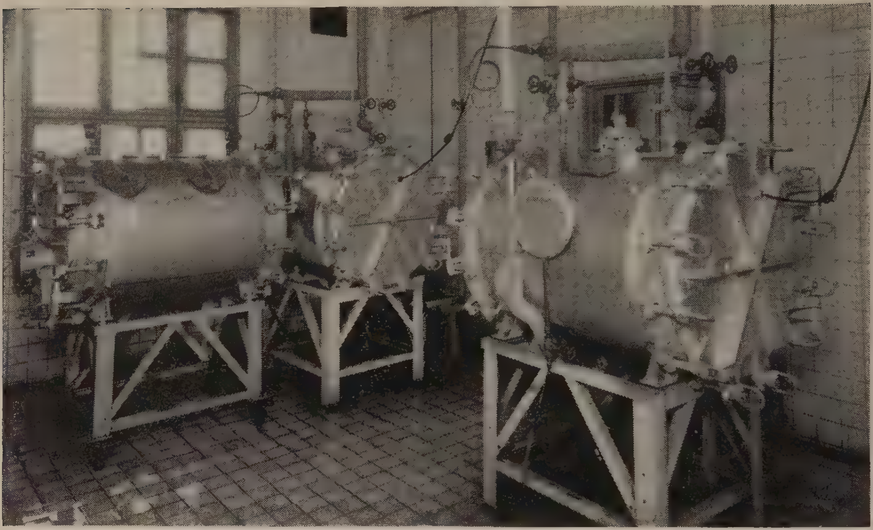

FIGURE 1. Three M-51 germfree rearing units in operation.

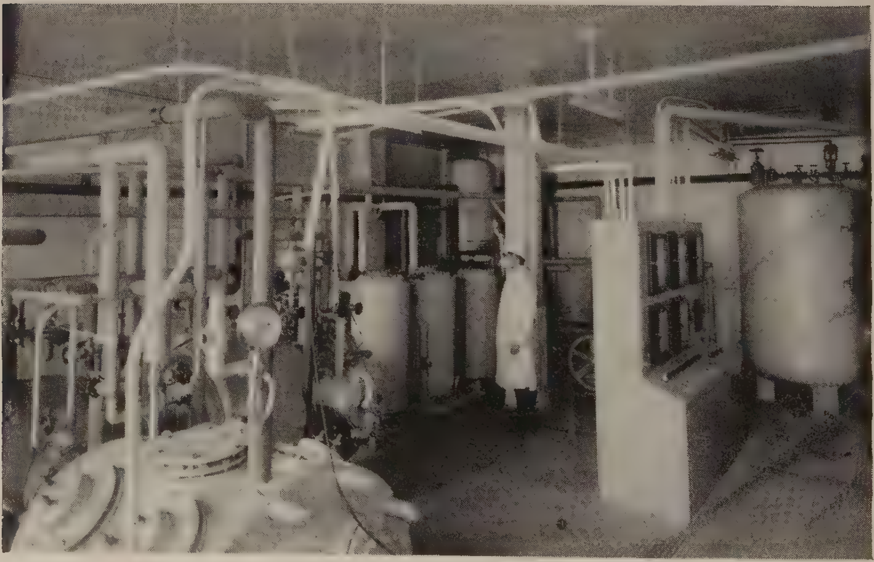

FIGURE 2. M-56 germfree rearing system installation.

by outside operation. The pan with the infant animals is pulled into the rearing unit with the remote control apparatus and carried into the middle part of the unit. The infants are picked up again one by one by the remote-control apparatus and put into rearing cages in the unit (FIGURE 5). The animals are wiped with gauze and are massaged at the anus and external orifice

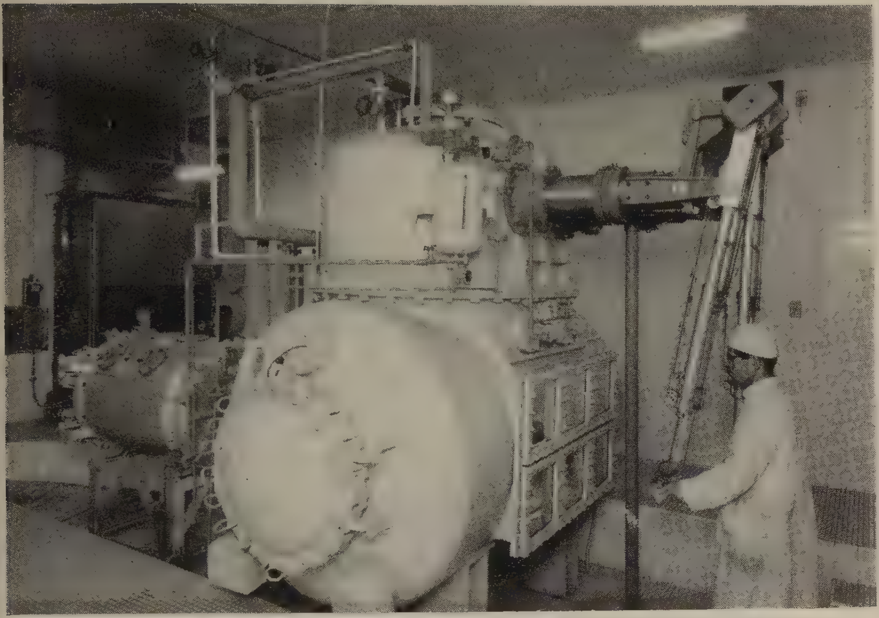

FIGURE 3. M-57 germfree rearing unit, showing remote-control apparatus.

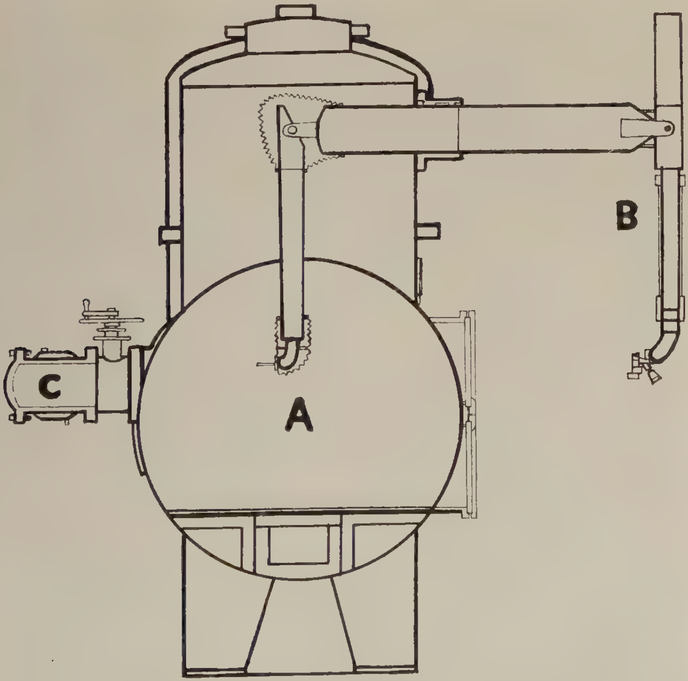

FIGURE 4. Diagram of the M-57 germfree rearing unit. *A*, germfree rearing room; *B*, remote-control apparatus; *C*, food-intake apparatus.

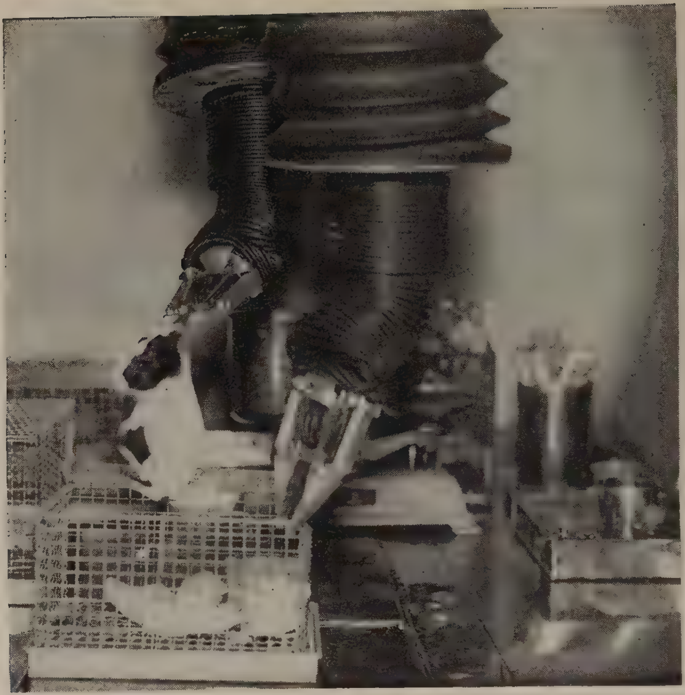

FIGURE 5. Guinea pig being inserted into a cage by the manipulators.

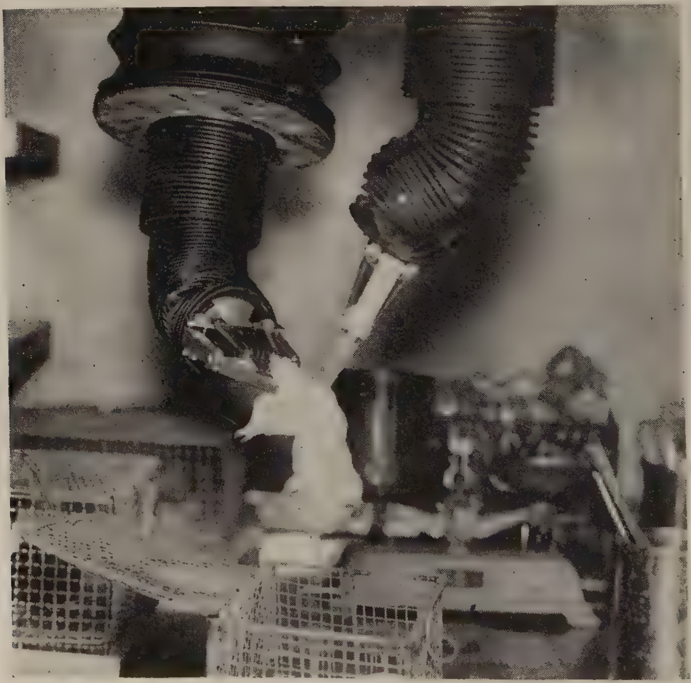

FIGURE 6. Massaging the anus and external orifice of the urethra of a germfree animal by means of the manipulators.

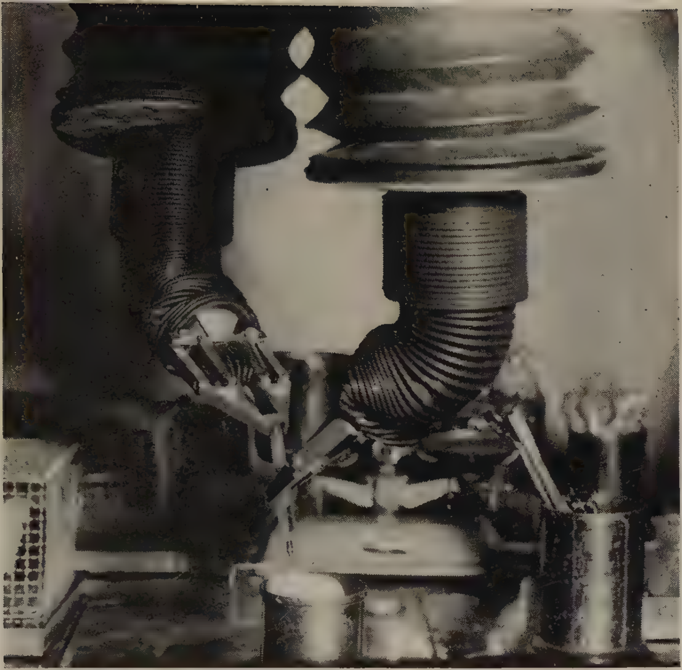

FIGURE 7. The injection of liquid vitamin into a germfree animal (in canister) by mean of the manipulators.

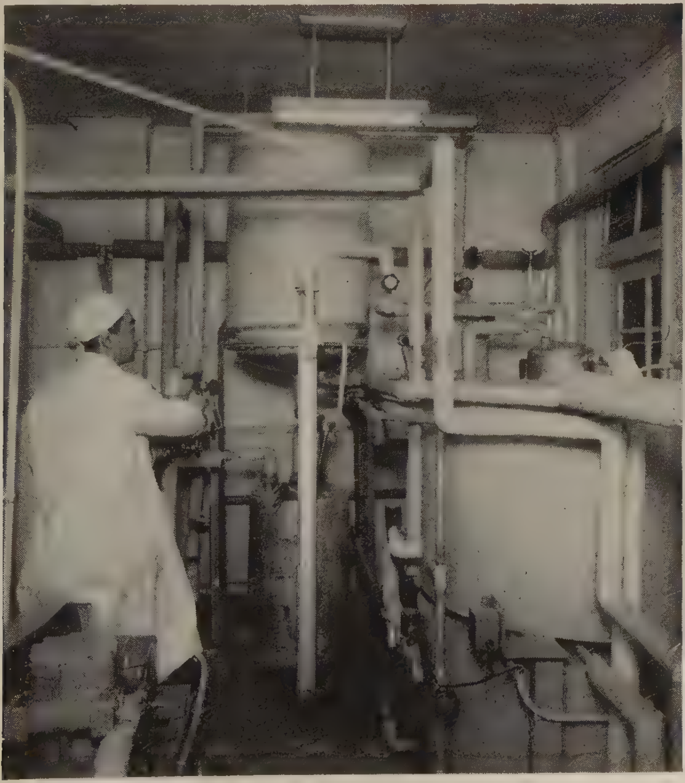

FIGURE 8. Steel humidity regulator in operation.

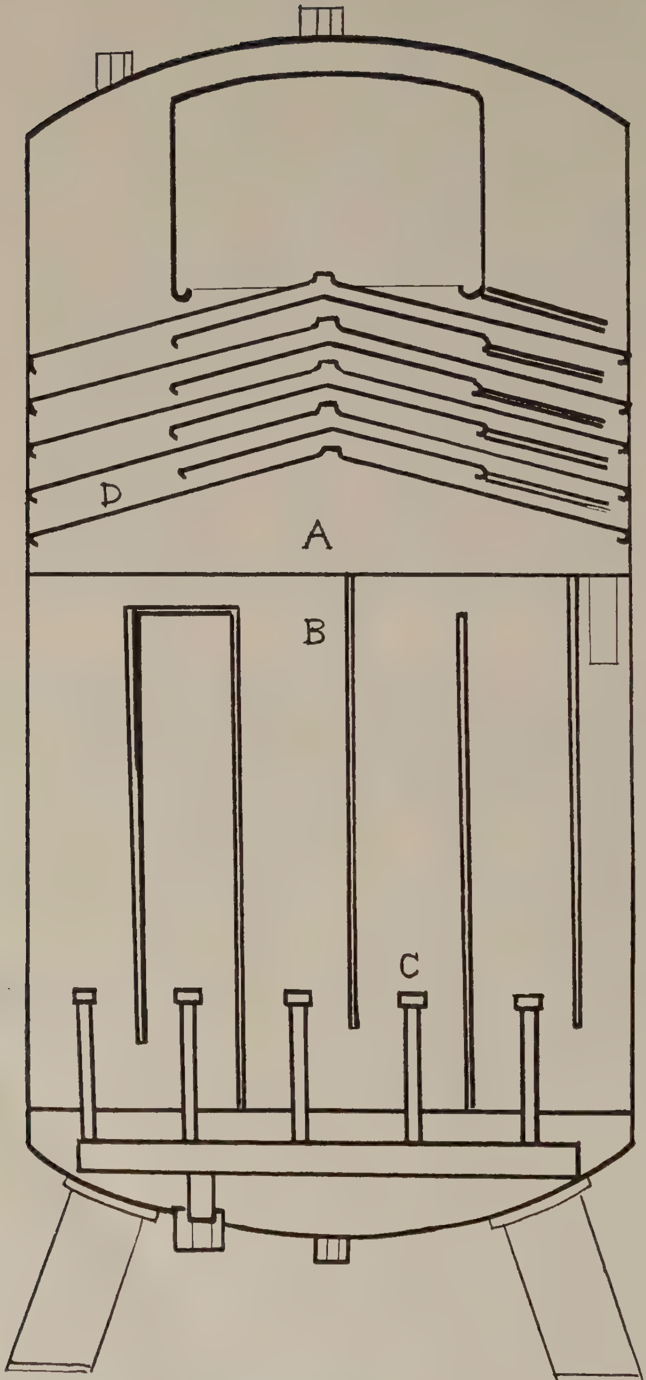

FIGURE 9. Diagram of the steel humidity regulator (FIGURE 8): *A*, upper chamber; *B*, lower chamber; *C*, spray nozzles; *D*, buffer plates.

of the urethra for the purpose of making excretion of feces and urine possible (FIGURE 6). By applying specially prepared food on the lips of the young they are stimulated to eat. The remote-control apparatus is satisfactory in measuring body weight and in making bacteriological examinations. This apparatus is versatile enough to attach a needle to a 1-cc. syringe, aspirate liquid vitamin from an ampule, and inject the liquid into a germfree animal (FIGURE 7).

Temperature in the rearing unit is automatically regulated constantly at 21 to 22° C. by an air conditioner. Humidity is also regulated automatically by a humidity regulator (FIGURES 8 and 9). The steel humidity regulator is divided into 2 chambers, upper and lower. Heated compressed air is passed through partitioned spaces in the lower chamber of the regulator. At the bottom of the lower chamber there are many nozzles through which water is forced at high pressure, thus forming a fine spray. This spray cools the air, which passes from the lower chamber to the upper chamber, in which numerous buffer plates are arranged. When the circulating air comes into contact with the refrigerated buffer plates the dew point is immediately reached. The vapor forms water drops that fall to the floor of the upper chamber and are drained out through special pipes. The air remaining in the upper chamber is then dehumidified, its humidity reduced to 50 per cent.

Six air filters and one air-heating apparatus are employed in order to obtain germfree air (FIGURES 10 and 11). The air filter is a double-walled cylindrical steel tank 400 mm. in inner diameter and 500 mm. in outer diameter, and 900 mm. in height. Seven glass-fiber coils are packed into the inner tank of each air filter. Four of the 6 air filters are used to filter the air

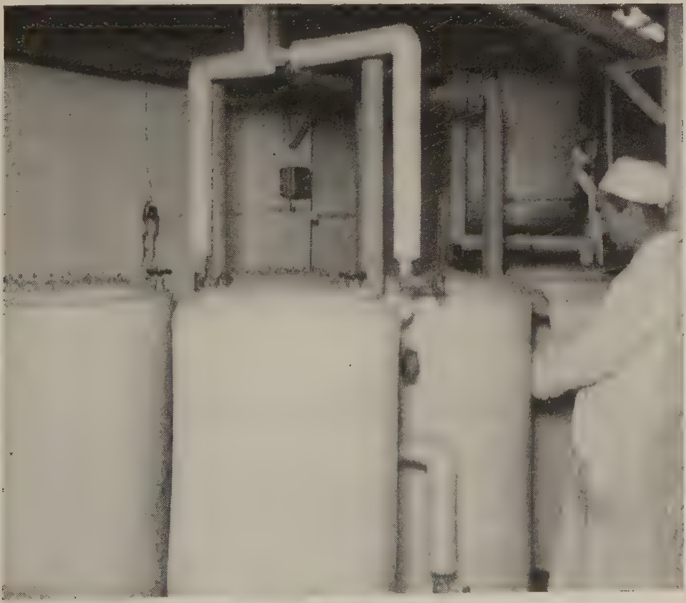

FIGURE 10. Air filters in operation.

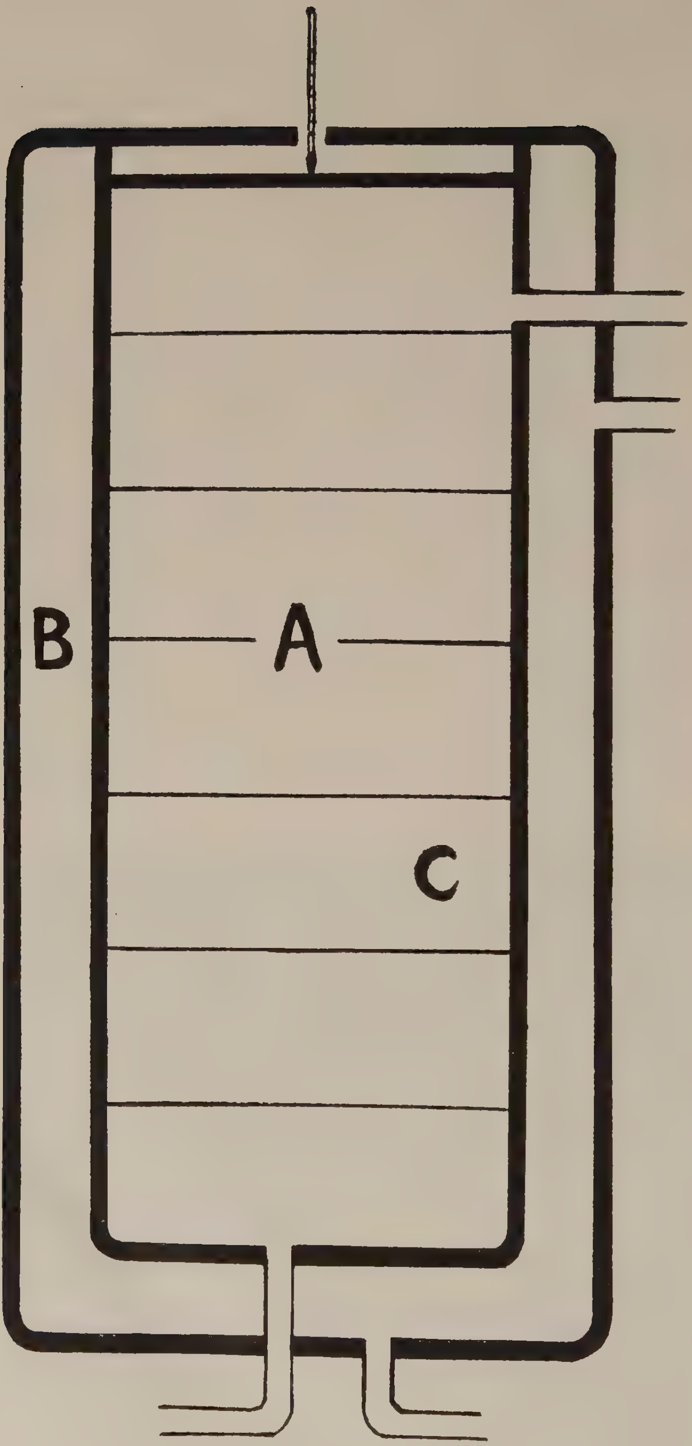

FIGURE 11. Diagram of air filter (FIGURE 10): *A*, outer tank; *B*, inner tank; *C*, glass-fiber coils.

that comes into the unit, the other 2 being located at the outlet. The glass-fiber coils are sterilized with steam under pressure every 3 days. In order to prevent damage to the glass fiber during sterilization, steam at 120° C. under pressure is passed into the space between inner and outer walls, and the temperature inside the inner tank where the glass fiber is packed reaches 90° C. in 10 min. The steam is then passed directly into the inner tank until the temperature there reaches 120° C., and this level of temperature is maintained for 20 min. The steam is then with-drawn from the inner tank,

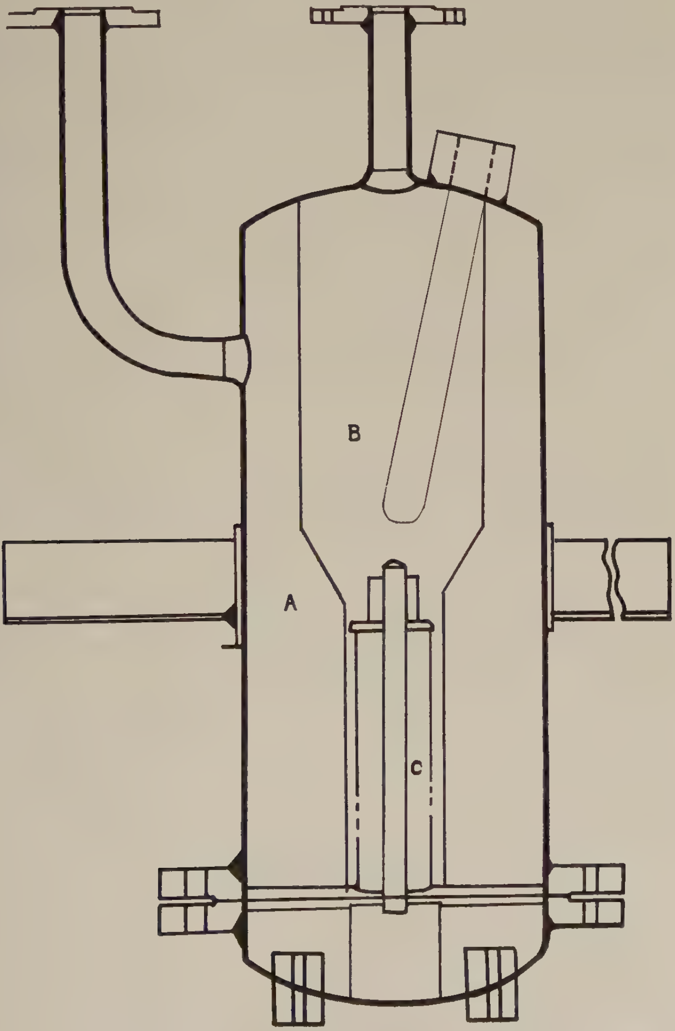

FIGURE 12. Diagram of air-heating apparatus: *A*, outer tank; *B*, inner tank; *C*, electric heater.

which is dried by the high temperature of the outer tank.

The air-heating apparatus is constructed of a double-walled steel tank and packed between the walls with magnesium, which serves as a heat insulator (FIGURES 8 and 12). The inner tank is divided into sections by vertical and horizontal partitions. An electric heater is placed in the center of the tank. The temperature of the air current reaches 400° C. as it is passed through the sections. This heated air, which is passed through pipes immersed in cold water, is cooled to about 25° C. and is finally introduced into the rearing unit.

Conclusion

The advantages of a rearing unit with remote-control apparatus are as follows: first, the size of rearing unit can be extended according to the size of the manipulator, whereas the size of a unit that requires rubber gloves is limited by the length of the gloves and of the arms of the operator. Consequently, greater numbers of animals can be reared in the rearing unit with the remote-control apparatus. Second, there is less possibility of contamination in the rearing unit with remote-control manipulators. Third, the manipulators are sufficiently versatile to perform fine maneuvers when handling inanimate objects. For all of these reasons various operations with the manipulators in the rearing unit can be learned even after a few days' training.

Two problems remain to be discussed. First, there is the difficulty of grasping a living animal that tries to escape from the mechanical hands. We have solved this problem by placing a set of buttons on the artificial fingers of the controls. By pressing these buttons we can increase the pressure exerted by the mechanical fingers. Second, there is the weak point caused by the use of silicon rubber on the joints. A device that enables outside joints to be immersed in germicidal fluids so that inside joints may be left uncovered is now being elaborated.

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DESIGN AND OPERATION OF APPARATUS FOR REARING GERMFREE ANIMALS*

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INTRODUCTION

Rearing germfree animals and using them in experiments is necessarily a problem in biological engineering; without apparatus that will eliminate contamination and at the same time permit animals to be maintained in it there is little point in attempting such experiments. The basic principles involved in the isolation of animals have been discussed elsewhere (Reyniers, 1943). What has not been discussed in any detail is the basic design, operation, and function of germfree apparatus. It is my purpose here to describe one such system, the Reyniers Germfree System II, and the results obtainable from its use.

The need for germfree animals is increasing. Many installations for their production are in operation, and more are presently anticipated. Consequently, it seems proper at this time to examine the steps involved in the operation of germfree equipment and the results obtained in order that the problems inherent in such work may be better understood. The fact that only one type of germfree apparatus is considered is satisfactory, apart from the limitations of space, for my purpose since the operational procedures are common to all such systems.

Until recently there was little point in attempting a critical appraisal of germfree operations, simply because the techniques for rearing such animals were not sufficiently advanced to be reduced to routines, and the necessary degree of certainty relative to contamination had not been attained. As long as the germfree animal remained a laboratory curiosity and the apparatus for working with it was not standardized, there was little purpose in such an analysis. The production of a few germfree animals was merely experimental, and the costs involved were relatively less important than if germfree animals had been available in numbers and widely used.

Highly skilled research personnel is no longer needed to operate germfree apparatus. Some species of animals can be reared germfree in numbers and maintained as colonies by breeding them through generations. Furthermore, this can now be done in a centralized establishment in much the same manner as in the commercial breeding of conventional animals. Shipment of germfree animals by public transportation to other centers is equally feasible (Reyniers and Sacksteder, 1958; Reyniers, 1957). Colonies of germfree animals can be tended by technicians, and the apparatus operated for experiments requires little more effort than does an autoclave. It is time, therefore, to discuss the operation of such equipment in terms of manpower, capacities, maintenance, and output of animals.

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The Reyniers Germfree System II is made to be sterilized *in situ* with steam under pressure. It should be noted that, if the need arises, this same system may be operated with chemical sterilization or by a combination of steam and chemicals. This system has been under development for more than twenty years, and with time and use it has been simplified and standardized. It is commercially available.*

The data given in this report are based upon the operation of three groups of this equipment, one used with rats and two strains of mice, one with guinea pigs, and the other with chickens. The same type of equipment has also been used to rear rabbits, monkeys, chickens, turkeys, dogs, and other animals. However, since the operation of germfree apparatus is essentially the same for any species, there is little point in discussing each species separately.

Basic Design of Germfree Apparatus

The Reyniers Germfree System is made up of a number of individual units called isolators, which can be used either separately or attached to each other to form multiple isolators. Sterilization of the isolator as well as of the supply lock is accomplished *in situ;* that is, the unit it is not sterilized separately in an autoclave or pressure vessel before use.

The basic Reyniers Germfree Isolator (FIGURE 1) is conventionally a cylinder to which are attached: a window, a pair of rubber gloves, a supply lock (also called a food autoclave), inlet and outlet filters for ventilating the isolator, openings for the steam, a drain, electricity, a stand that permits the isolator to be moved from place to place, and a door used to close the open end of the cylinder.

The standard one-man isolator is 28 inches in diameter and 46 inches long. This size is determined by the maximum interior area that can be reached through the rubber gloves from a standing position. The isolators are 42 inches from the floor to midline.

The length and diameter of a germfree isolator may be changed without affecting the basic design. If the isolator is increased in diameter or made longer it must be operated with additional pairs of gloves with a window to each pair. In this way germfree isolators have been built as large as 36 inches in diameter and as long as 12 feet.

The cylindrical shape commonly used permits greater economy in machining the parts and is strongest for the purpose. However, it is possible to use a rectangular or semiround section without changing the basic design. The operation of these isolators is the same regardless of their shape or size.

The standard basic isolator has been modified to four specialized types (FIGURE 1) that experience has shown generally to meet most experimental needs. These specialized germfree isolators are: (1) rearing isolator (RSU-)† used for rearing and breeding animals; (2) examining isolator (REXU-)

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* Reyniers and Son, Chicago, Ill.

† The code letters are those of Reyniers and Son and designate the type of germfree isolator; the number following the letters designates the series, which may differ in size or construction details. For example: RSU-400 is a standard stainless steel rearing isolator 28 inches in diameter and 46 inches long.

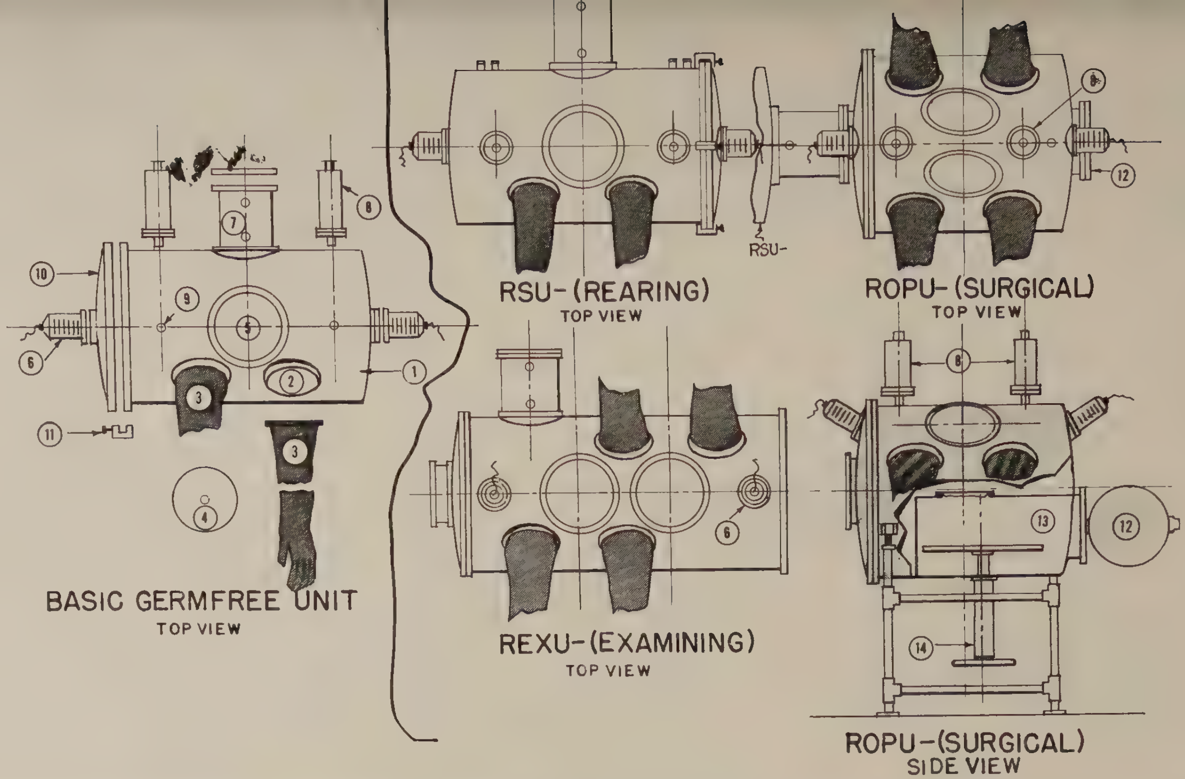

Figure 1. Basic and specialized germfree isolators.

Basic isolator: (1) Shell; (2) glove ring for attaching gloves to germfree isolator; (3) rubber gloves; (4) lids for closing glove rings during sterilization (these lids are fitted with a valve for regulating pressure in gloves); (5) window of Pyrex glass; (6) external lamps fitted with ventilating fan and detachable from the light window; (7) double-walled supply lock fitted with 2 quick-opening doors; (8) air filters (traps may be substituted for these); (9) openings in isolator for filters (there may be many such openings on a germfree isolator); (10) end door for closing isolator; and (11) C-clamps for attaching end door.

Specialized isolators: RSU-rearing isolator; general purpose isolator; REXU-examining isolator, intended primarily for experimental work requiring 2 operators and for combining with other germfree isolators to form a system; and ROPU-surgical isolator, intended primarily for cesarotomies; (12) quick-opening door closing off animal compartment on ROPU-surgical isolator; (13) animal compartment closed from the isolator interior by a sheet of cellophane; (14) mechanism for raising pregnant animal into contact with cellophane barrier.

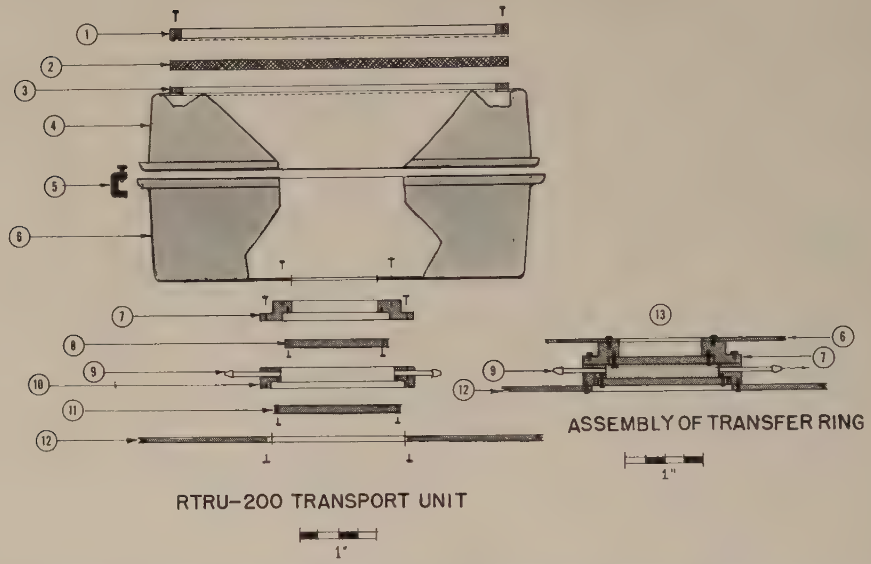

FIGURE 2. Germfree transport isolator. (1) Top frame with one-eighth-inch mesh screen; (2) FG-50 glass-fiber filter mat; (3) bottom frame with protective screen for filter mat, which is compressed between the frames; (4) top of aluminum roasting pan; (5) C-clamps for fastening top to bottom against a rubber gasket; (6) bottom of aluminum roasting pan; (7) A-ring bolted to bottom of roasting pan against a gasket; (8) plate for closing A-ring; (9) pipe for filling span between A- and B-ring with germicide; (10) B-ring, which is bolted to a sterile transfer isolator; (11) plate for closing B-ring; (12) wall of transfer isolator; (13) germicide.

usually made with two pairs of gloves and two open ends*; (3) surgical isolator (ROPU-) mounting two pairs of gloves and intended primarily for cesarotomies, although other surgical procedures may be performed in it; (4) transport unit (RTRU-) in which germfree animals may be shipped by public transportation (FIGURE 2).

Any or all of the isolators mentioned may be joined to form experimental systems (FIGURE 3). If the isolators are connected by supply locks, they may be taken apart or assembled without affecting sterility; if the isolators are attached end to end this cannot be done.

Two general types of large-scale germfree isolators have been designed for use with large animals or large numbers of smaller animals. One such system (RCOL-1) permits the attendant, clothed in a plastic garment, to enter it (FIGURE 4); the other (RSTU-1, shown in FIGURE 5), allows a number of animal pens to be brought to a station where the technician can tend the animals by means of rubber gloves. This system, in a smaller form, has been used as a storage system for animals. There are many modifications of these basic types, all of which have been described elsewhere (Reyniers, 1956, Reyniers, patents).

Over the many years during which this apparatus has been developed it

* This isolator may be attached to other isolators, end to end, or via the supply lock to make a system in which many experiments requiring two or more technicians can be performed.

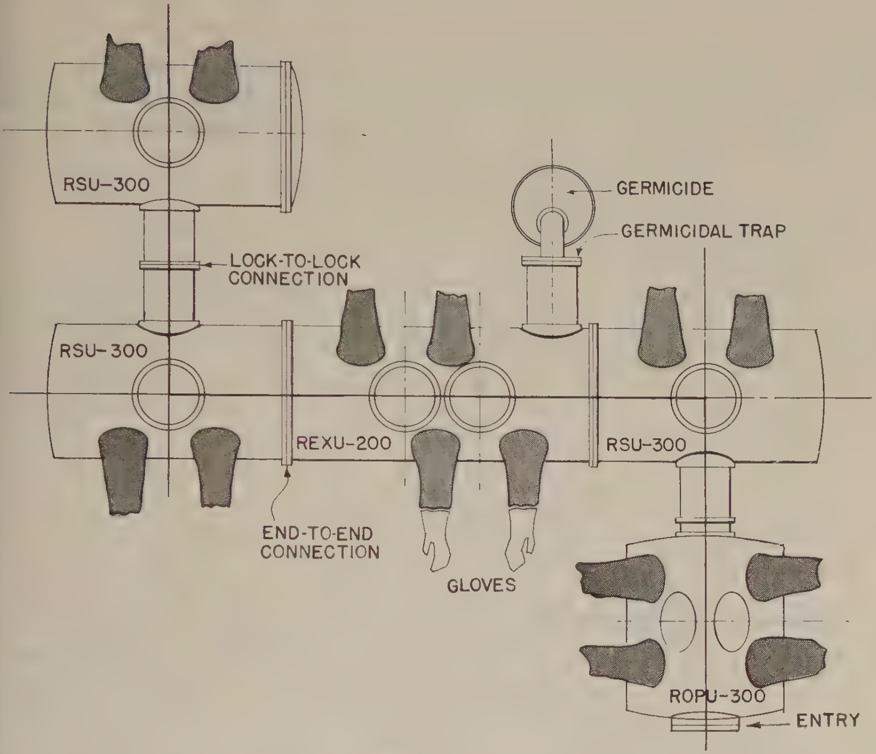

FIGURE 3. Assembly of germfree isolators into experimental systems. The units can be combined to form various combinations involving many operators. The assembly shown is intended to demonstrate combinations by means of supply locks and end-to-end combinations. Entry to the system is by way of the surgical isolator, the various supply locks, and through a germicidal trap. Combinations through the supply locks permit the isolators to be separated without contamination.

has been constructed of brass, of mild steel with painted finishes, of stainless steel, and of aluminum. The metal of choice is stainless steel, which can be polished and is easily maintained. The materials used do not affect the basic design. However, since this equipment has been operated safely for from twenty to twenty-five years, stainless steel has become standard. It is also standard practice to build the shell from 12-gauge (0.081-inch) metal with equally sturdy machined elements. Heavy construction stands up better to the routines of heating and cooling and of moving the equipment from place to place. Weight is not important, since the isolator is moved on ball-bearing wheels. The isolators are easily moved and can be operated by women. Experience has shown that light construction will not withstand the strain of repeated heating and cooling and day-to-day use.

OPERATION OF GERMFREE APPARATUS

All germfree equipment, irrespective of design or kind, is operated essentially in the same way and involves the same steps. Much of the cost of working

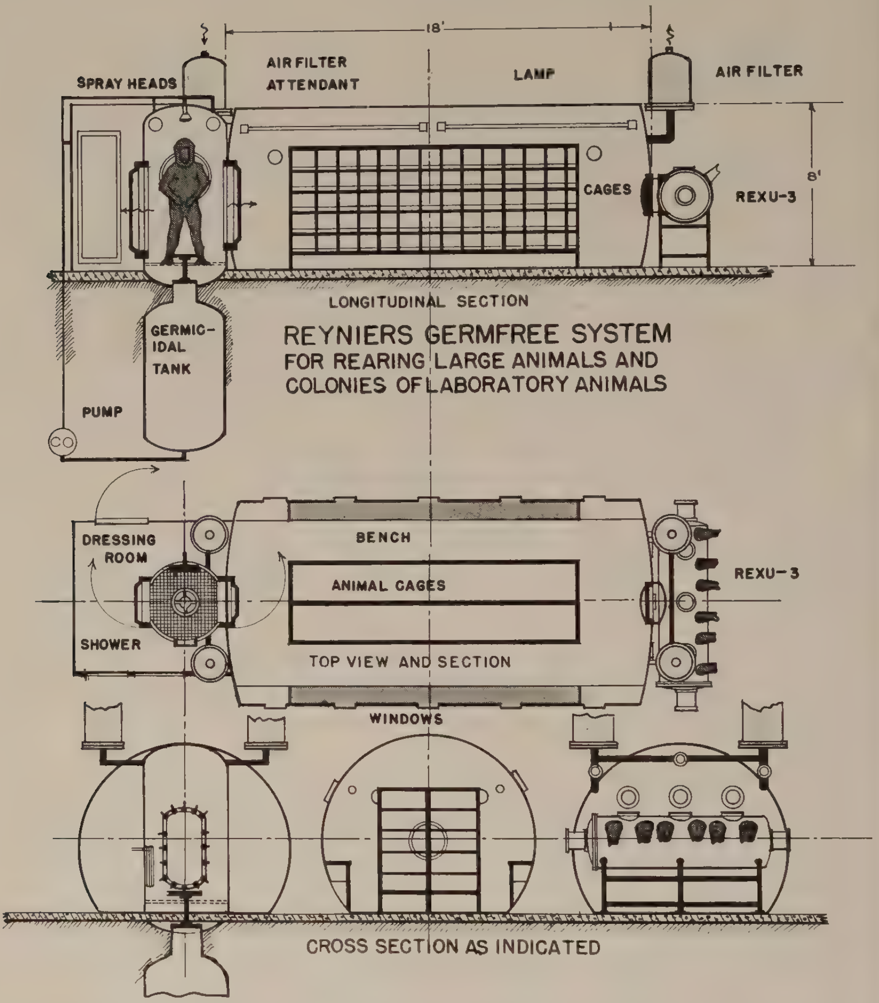

FIGURE 4. RCOL-1. This isolator is intended for rearing large animals or large numbers of animals as a breeding colony, or for using large numbers of germfree animals in experiments demanding the same environment. This diagram illustrates the isolator as it was originally designed. It consists of a tank 18 feet long and 8 feet in diameter, constructed of stainless steel sufficiently heavy to withstand steam sterilization. This tank is equipped with 2 doors, one attached to an REXU-examining isolator and the other to the entry lock. The entry lock is enclosed by a dressing room and can be sterilized with steam. In operation the attendant is sealed into a plastic garment supplied with air inlet and outlet and telephone communication. On entering the lock, the suit is sterilized by sprays of detergent and germicide. It is possible to flood the lock entirely with germicide and so immerse the attendant. Once the suit is sterilized, the attendant enters the main tank and tends the animals. This operation is on one level; because of the lock, quick-acting germicides may be used. The interior of the tank is equipped with standard animal cages. Ventilation is accomplished through large filters, which are in duplicate so that they may be sterilized while the unit is in operation.

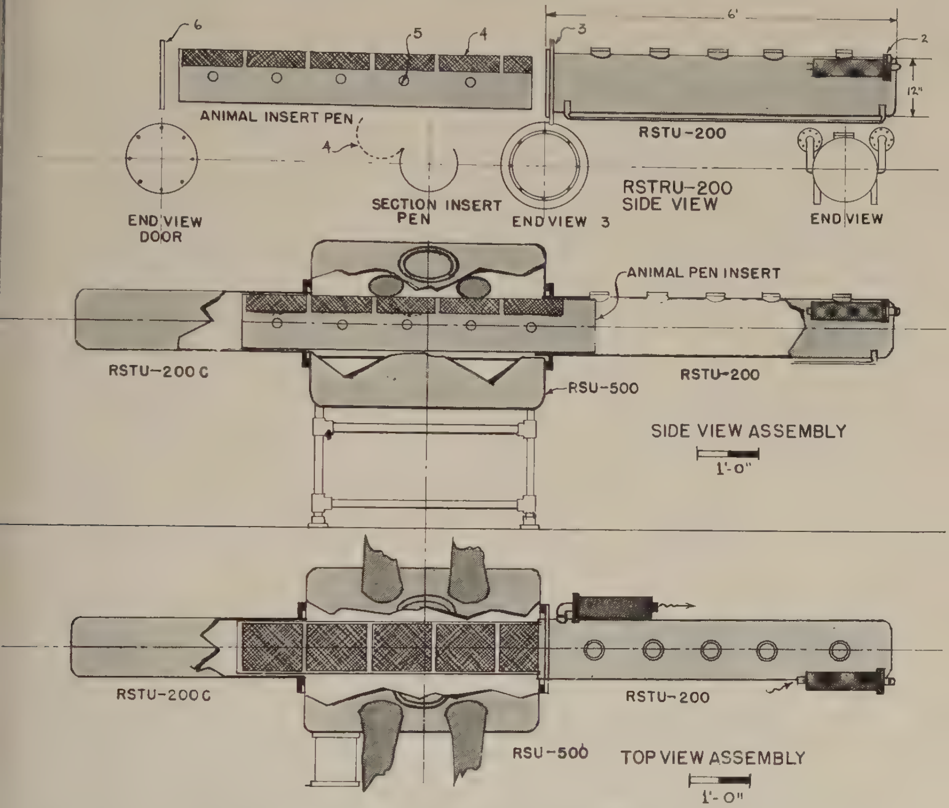

FIGURE 5. RSTU- one-station germfree colony system. This system makes use of cylinders that can be stacked. Each cylinder (RSTRU-200) contains an animal pen insert with separate compartments. In use, the RSTRU-200 isolator is bolted to an RSU-500 isolator with an adapting end door, and an RSTU-200 C-isolator is fastened in the opposite end. The assembly is sterilized. The door to the RSTRU-200 isolator is opened, the animal insert pen is pulled into the RSU-500 isolator and on into the attached RSTU-200 isolator. Pens can be cleaned, animals examined, and food supplies received. The sections of the insert pen: (1) windows; (2) air filter; (3) flange for bolting RSTU-200 to the RSU-500; (4) screen access doors; (5) ventilators; and (6) door for closing RSTU-200 isolator.

with germfree animals is in operation and use of the equipment. If the apparatus is not dependable and an experiment becomes contaminated, the costs are relatively greater. The principal expense is not, as might be thought, in the initial cost of the apparatus, since this may be spread over many years, but in the reliability of the apparatus during use.

Assembling and Testing

At the start or completion of an experiment, a germfree isolator must be disassembled and cleaned. The end door is removed, the contents of the unit removed, and the inner door of the supply lock opened. All inside surfaces of the isolator, the apparatus used in it, and outside surfaces are washed with a detergent. We believe that careful cleaning is an important preliminary to complete sterilization.

When the isolator is reassembled it is routine to replace the rubber gloves and to examine and replace all gaskets that show compression or wear. Valve diaphragms are replaced at each initial sterilization. After several years' use the standard Reyniers Air Filters may be repacked with glass fiber, although we have run these isolators for ten years or more without doing so. On the other hand, the glass-fiber mat or cloth used in the Trexler Air Filters must be changed before each sterilization. When the end door is fastened a little care in drawing it tight against the gasket will avoid leaks. It seems almost elementary to point out that this is best done by equally tightening the clamps opposite each other.

The next step is to test the inner door of the supply lock for tightness by closing both doors and testing the lock with 20 p.s.i. of air. When the supply lock proves to be tight the pressure is relieved, and the inner door opened.

The final test is on the main body of the germfree isolator. This is done by filling the isolator with 20 p.s.i. of air and allowing it to stand overnight. Any decrease in pressure, noted on an attached pressure gauge, indicates a leak that must be located. This is done by painting a viscous soap solution on the gasket edges and valve diaphragms at suspected spots, edges, and pipe joints. If bubbles form the leak can be located and stopped. In most instances this can be done simply by drawing the gasket surfaces tighter. Increasing the air pressure will shorten the time for detecting leaks, just as will the use of electronic leak detectors, but the use of soap solution is readily adapted to routines and has proved to be generally satisfactory.

There are not many places where a leak can develop on a standard germfree isolator. All pipe joints are silver soldered, and the window seal, once it is tight, usually lasts the life of the isolator, or at least for many years, and seldom requires further attention. The glove seals occasionally may show leaks when the gloves are changed, but this can be corrected easily by tightening the holding ring. The end door and outer supply lock door already have been considered.

A check of the records of the operations reported herein shows that very little time has been spent on assembly leaks. For example, in an average series of 15 assemblies only one was found, and this was on an end door. This condition was corrected by tightening the bolts at the point of leak. If proper care is exercised in assembling the isolator there should be little trouble with leaks.

In checking for leaks it is our custom to allow the isolator under test to remain overnight. Since we do not sterilize at night, we have found a satisfactory routine of placing several isolators on test at one time at the end of a working day.

As a matter of record it takes from 4 to 6 hours to disassemble, clean, reassemble, and repair detected leaks in the initial sterilization of a single germfree isolator. While the time can be shortened, we have found that it is best to perform this step carefully, since the success of the experiment depends upon it.

Initial Sterilization

Once a germfree isolator is free of leaks it is ready for sterilization. At this point it is connected to the necessary services through flexible hoses attached to a sterilization cart (FIGURE 6). The services are: steam, drain, vacuum, air, and water. A recorder on the sterilization cart permits a time-temperature record of the sterilization cycle, and this is filed for reference.

Before sterilization an insulating blanket is spread over the entire germfree isolator covering all the exposed surfaces, including the ends and the supply lock, except for an opening over the window. It is necessary to be able to observe the rubber gloves at all times. The insulating blanket is made of two layers of glass cloth between which a pad of glass fiber 2 inches thick is quilted, using glass fiber thread for sewing. The blanket is held in place with straps and drawstrings. The use of an insulating blanket ensures better sterilization and reduces the heat in the room.

Temperatures are taken with thermocouples inserted into the drain and, when the occasion demands, into other areas. FIGURE 7 shows the valves and the germfree isolator at sterilization.

The eight steps in the procedure for sterilization are as follows:

(1) A vacuum of 20 inches Hg is maintained in the isolator for 10 min. Pres-

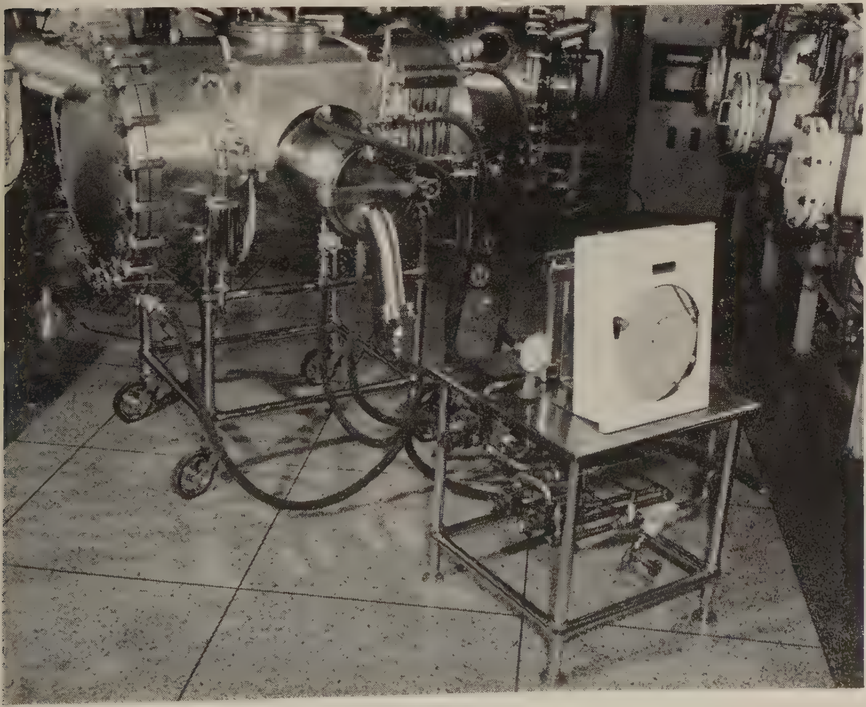

FIGURE 6. Sterilization cart attached to an RSU-300 isolator.

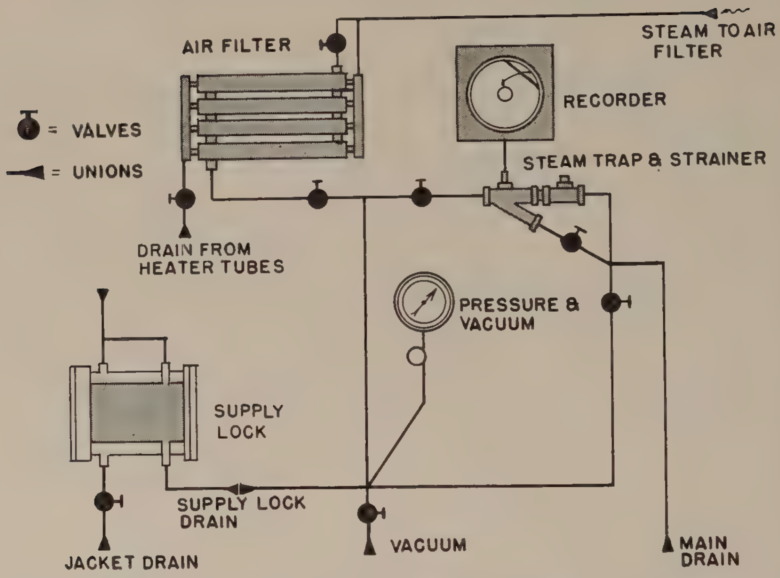

FIGURE 7. Schematic diagram of the piping in the type of sterilization cart used in Group I studies in this report.

sure is equalized on the rubber gloves by small valves in the glove port covers. It is important that the gloves not be allowed to collapse or distend completely at any time during the sterilization cycle. They are best kept partially inflated to prevent creasing.

(2) The vacuum is broken by admitting freely flowing steam into the isolator, to the jacket of the supply lock, and to the air filters until the temperature reaches 212° F.

(3) Steam pressure is now built up to 252° F., always watching the rubber gloves to prevent them from collapsing. When the temperature reaches 252° F., it is held there for 30 min.

(4) At the end of this period the pressure is broken, and a vacuum of 20 inches Hg is created in the isolator. The vacuum is created by steam injectors, since it is necessary to remove the condensate that forms.

(5) The vacuum is held from 10 to 15 min., which is usually adequate to dry out the filters and the isolator interior.

(6) The air filters, which are also sterilized *in situ* with the germfree isolator, are supplied with additional heat through electric heating elements or a steam coil to ensure drying the glass fiber when the vacuum is created; a wet or moist air filter is useless for sterilizing air.

(7) The vacuum is then broken carefully and continuously by admitting air through the filters until the pressure is normal. The insulating blanket is then removed and the isolator, when it is cool, is ready for use.

(8) After sterilization the isolator may again be tested for leaks and, if tight, the glove port covers are removed and the inner door to the supply lock closed.

Placing Animals in the Germfree Apparatus

Animals may be placed into the germfree apparatus as: (1) newborn from a cesarotomy or from eggs that have been incubated to hatching in the supply lock, (2) from another isolator already containing germfree animals, or (3) from a transport isolator.

If newborn mammals are placed in a germfree isolator (RSU-), it must first be attached to a sterilized surgical isolator (ROPU-) by means of the supply lock. The technique for cesarotomy and hand feeding germfree mammals has been described elsewhere (Reyniers *et al.*, 1946). If chicks are to be hatched in a germfree isolator, the eggs are passed through a germicidal trap (FIGURES 8 and 9) and incubated in the supply lock. The technique for hatching and rearing germfree chickens has been described elsewhere (Reyniers *et al.*, 1949*a* and 1949*b*).

If germfree animals are to be transferred from another germfree isolator, the two isolators are attached by means of the supply lock, and the linkage is tested, then sterilized.

Animals may be transported from one place to another by public transportation (Reyniers and Sacksteder, 1958). The germfree isolator used to accomplish this is shown in FIGURE 2 and consists of a box fitted with a free-

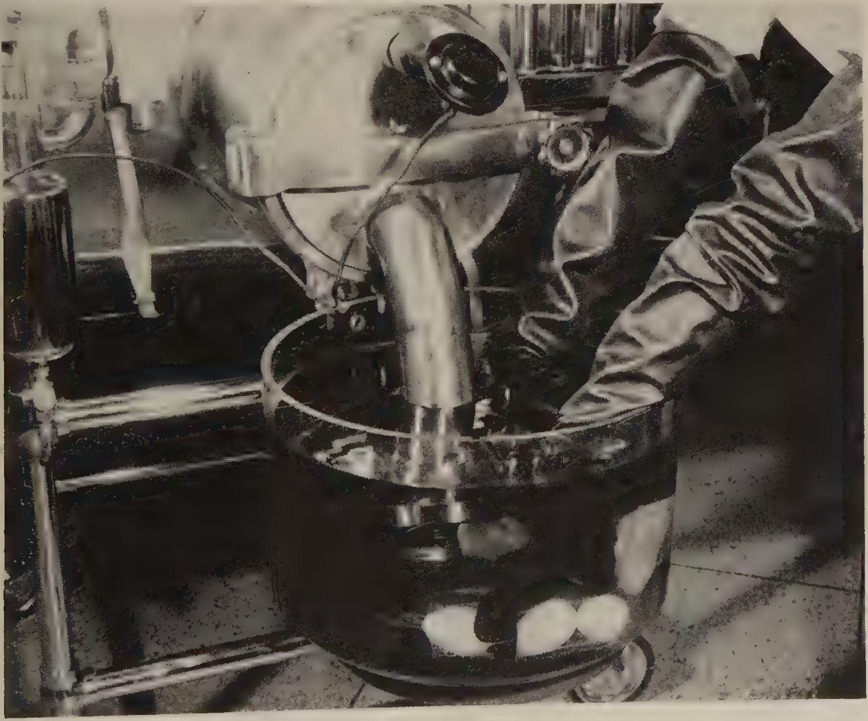

FIGURE 8. Germicidal trap attached to a germfree isolator.

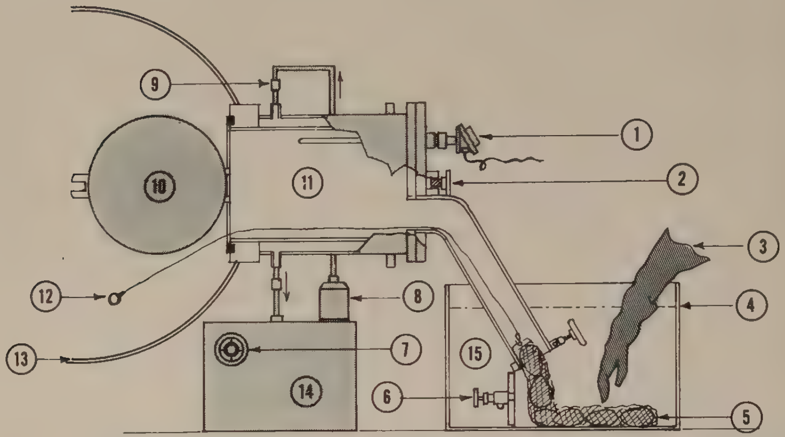

FIGURE 9. Diagram of a germicidal trap attached to a germfree isolator: (1) thermoregulator; (2) standard quick-opening door; (3) rubber gloves for opening passage tube door and inserting eggs or other materials; (4) level of germicide; (5) hen's eggs placed into a nylon mesh tube fastened to the hook of a pull chain; (6) valve on door of passage tube to bleed condensate during sterilization; (7) thermoregulator; (8) pump; (9) union connecting tempered water to outer jacket of supply lock; (10) inner door to supply lock; (11) supply lock; (12) pull chain for bringing materials into the germfree isolator; (13) wall of germfree isolator; (14) water bath for regulating temperature in supply lock; (15) Pyrex or stainless steel vessel.

flow air filter and a transfer ring that forms a germicidal trap. The transport isolator (RTRU-) is first placed in a rearing isolator (RSU-) and sterilized. It is then attached to an isolator containing germfree animals, and the linkage is sterilized. The animals are then removed from the colony isolator and placed in the transport isolator (RTRU-), which is sealed shut and removed from the isolator (RSU-). It may now be shipped by truck, rail, or air. When it arrives at the point of use it is attached to a germfree isolator by the retaining ring. The inner space formed between the bottom of the transport isolator and the top of the receiving isolator is sterilized with germicide. The doors or plates are removed, and the animals are taken into the germfree isolator for experimental use.

Supply Lock Operation

All food, water, bedding, apparatus, and such supplies as syringes, swabs, culture media and surgical instruments are sterilized *in situ* in the supply lock. All wastes, such as soiled bedding, empty containers, and dead or living animals are removed from the germfree isolator through the same device. As previously noted, the supply lock is also used to make transfers from isolator to isolator and may also serve as a passage into the isolator through an attached germicidal trap. With minor variations, the operations performed are essentially the same, so that it is necessary to detail only the procedure for taking food and water into the germfree isolator.

Perhaps it should be noted at this point that a sterilized supply lock is maintained at all times on every germfree isolator in use so that animals may be removed from it. The outer door is never open longer than necessary to place materials into the lock or remove them.

The steps necessary to proper supply-lock operation are as follows.

Preparation of supplies. Presterilized No. 2 milk cans are filled with boiling water through a hole in one end, which is then soldered shut. Screw-top containers are generally avoided because they are difficult to sterilize. If they must be used, then the gasket and cap should be separated before sterilization.

All liquids, such as diets and vitamins are sealed in previously sterilized (dry heat) glass ampules. The surface of the ampules should be washed in a detergent before sterilization. Dry nonpelleted diet is loosely packed in cloth bags, which are flattened to one-inch thickness and laid on wire screen to ensure penetration of steam. Wood shavings, used as bedding, may be packed in the same way and in the same type of bags used for diets. Pelleted diets are sterilized on a wire screen. All materials, such as wood shavings, that will withstand repeated sterilization should be presterilized.

Presterilization. It is especially important to remember that in germfree work all living organisms, not only in the food or other supplies, but also on the surface of all containers, the inside of the supply lock, inner surfaces of doors, and the contained air, must be killed. In order to ensure sterilization the exposed surfaces of the inside of the supply lock are carefully washed with a detergent, and 2 per cent alkyl aryl quaternary ammonium chloride (Hyamine) is sprayed on the gasket surfaces of the doors. In other words, before the supply lock is packed it is carefully washed. Care in this detail ensures successful operation over long periods of time and through many hundreds of supply-lock passages.

The carrier (FIGURE 10) into which the supplies are packed fits into the supply lock, leaving a space of about one-quarter of an inch around it. The bottom is perforated, and a wire screen separates it on the long axis. Previous to use this carrier is soaked in a hot detergent and carefully scrubbed with a brush to free it of all debris, after which it is soaked in 2 per cent Hyamine.

Sterilization. After the supplies are packed into the carrier it is placed in the supply lock and the outer door closed. A vacuum of 20 inches Hg is created in the lock and held for 10 min. to test for tightness. Free-flowing steam is then admitted to the jacket of the supply lock until a temperature of 212° F. is registered. Steam is now admitted to the interior of the lock until a temperature of 212° F. is reached. At this point the drain is throttled down and steam pressure built up in both the jacket and the isolator interior to 252° F. for 25 min. At the end of the sterilization cycle a vacuum of 20 inches Hg is created in the supply lock, and steam is allowed to circulate at 252° F. in the jacket for 10 min., which is usually sufficient to dry out the contents. At the end of the drying cycle the negative pressure is relieved with air passed through an air-sterilizing filter mounted on the sterilization cart. Immediately after cooling, the inner door of the supply lock may be opened and the contents removed.

During sterilization of the supply lock it is necessary to avoid heating the

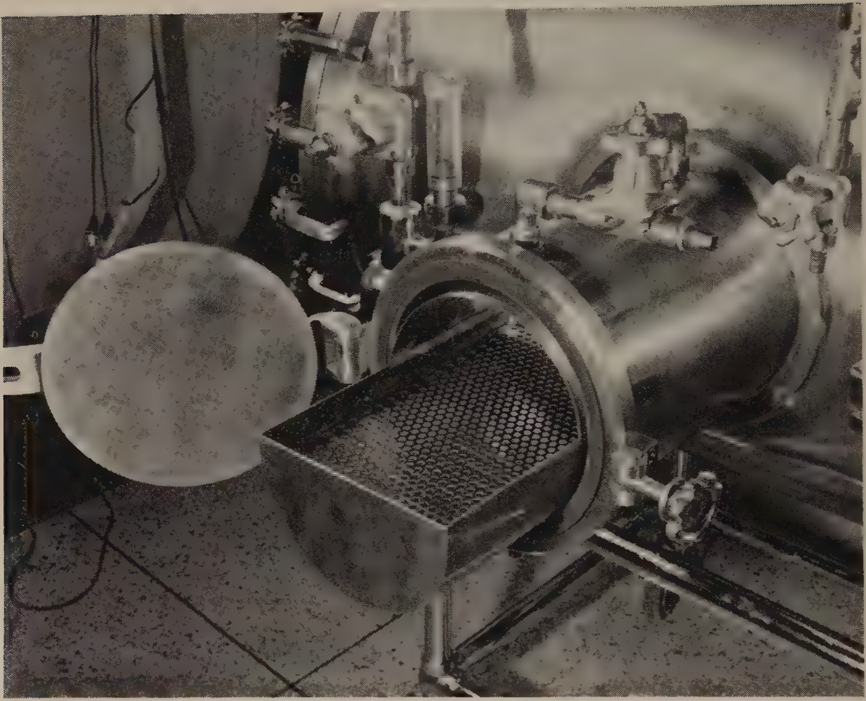

FIGURE 10. Supply lock, showing insert.

interior of the germfree isolator. This can be accomplished by passing a cooling stream of water over the outside wall of the germfree isolator about 2 inches from the outside casting that fastens the food autoclave to the wall of the isolator. Additional protection is obtained by using an insulating cap that fits over the inner door of the supply lock. This is made in the same way and of the same materials as the insulation blanket already described. It is sterilized at the time of initial sterilization or by passing it through the supply lock. With this protection and the normal ventilation (3.5 cubic feet per minute) of the stainless isolator, there is less than 1° C. rise in temperature at a distance of 6 inches from the inner door. At the position of the animals there is no appreciable temperature rise. As far as can be determined, repeated sterilizations of the supply lock have no effect on the animals in terms of growth and reproduction. This certainty as to the harmlessness of the sterilization process to the condition of the animals leaves little doubt as to the effectiveness of the procedures employed.

One point should be noted. Before opening a sterile supply lock to the outside it should be tested with air pressure to determine the tightness of the inner door. Air is supplied to the lock through sterile air filters mounted on the sterilizing cart.

Passage of Heat-Labile Materials into Germfree Apparatus

There are occasions when it is necessary to pass materials into the germfree apparatus without subjecting them to heat. This can be done by the use of a liquid germicidal trap, or by the use of germicidal vapors (ethylene oxide, peracetic acid; see Trexler and Reynolds, 1957) in the supply lock. In those instances when viruses are not important it is also possible to pass liquids through ultrafine filters attached to the germfree isolator and sterilized *in situ*. Examples are the passage of fertile eggs, antibiotics, pure cultures of microbes, animal sera, and some food supplements. In most instances the germicides are used to sterilize the surface of the containers holding the heat-labile material. We have used radiation (Luckey *et al.*, 1955) to sterilize food supplements and antibiotics. These materials are placed in a double plastic bag, which is passed into the germfree isolator through the liquid germicidal trap.

In the work reported herein only the liquid germicidal trap has been used. This trap (FIGURE 8) consists of a stainless steel tube 4 inches in diameter, welded at an angle into a supply lock door. The bottom end of the tube is fitted with a hinged lid that can be closed against a gasket by a screw acting on a bar. The door is furnished with a small valve for bleeding condensate during sterilization. The germicidal trap is used as follows: (1) the trap is fastened into place on a supply lock and tested for tightness; (2) the sterilization procedure is the same as already described; (3) after sterilization and drying, the bottom end of the 4-inch tube is submerged into germicide; and (4) in the case of fertile eggs the germicide of choice is 2 per cent $HgCl_2$ and the eggs are placed in a nylon mesh tube immersed in the germicide. The door on the end of the 4-inch tube is opened by hand below the surface of the germicide. A rubber glove protects the hands. A hook on the end of a stainless steel chain passing into the germfree insolator is fastened to the nylon tube containing the eggs, which are then pulled into the germfree isolator. The eggs are then taken from the mesh tube and incubated. The lid on the end of the germicidal trap is closed as is the inner door of the supply lock. The details of egg incubation have been described elsewhere (Reyniers *et al.*, 1949).

Sterilization of Air and Ventilation of Isolator

Insofar as can be determined, sterilization of air has caused us the least trouble. If the glass fiber air filters become wet, contamination will occur, but when this happens it is the fault of poor maintenance. Beginning with a complicated air-sterilization system in the early 1930s, we have simplified it to its present form.

Several styles of air filters have been used on the equipment described in this study. All, however, are essentially the same inasmuch as they all depend upon glass fibers and *in situ* sterilization with steam, followed by drying in a vacuum. The operation of these filters requires little attention and, as can be seen from the results reported, we have run such filters continuously for at least nineteen months without resterilization.

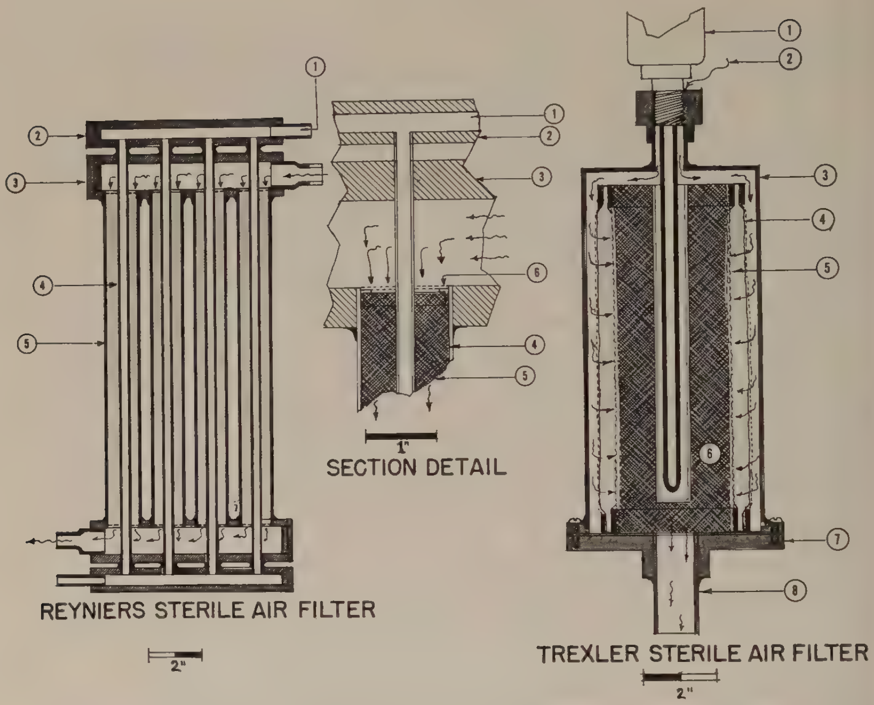

FIGURE 11. Design of air filters for use on germfree isolators. These air filters are sterilized *in situ* with steam under pressure and dried by heating in a vacuum. Left: cross-section of Reyniers Sterile Air Filter; (1) inlet for steam to heat glass fibers after autoclaving; (2) manifold for heating pipes; (3) manifold for filter tubes; (4) glass fiber, packed evenly and to a standard weight per volume; and (5) filter tubes. Center: cross-section detail of Reyniers Filter: (1) steam inlet; (2) manifold for heating tubes; (3) manifold for filter tubes; (4) filter tube; (5) glass fiber; and (6) screens at both ends of filter tube. Right, cross-section of Trexler Filter: (1) electric heating element for use during drying after steam sterilization; (2) T-inlet for air; (3) outer shell of filter; (4) wire screen; (5) two or more layers of glass fiber filter mat (AAF-FG-50) through which air must pass; (6) inner screen around which filter mat is wrapped; (7) base to which cover shell is bolted; and (8) inlet to isolator.

The Reyniers Air Filter offers more resistance to the flow of air than does the Trexler type, which employs a sheet or pad of glass fibers (FIGURE 11). On a basis of use, both seem to be equally effective. On the isolators where an outlet trap has been employed; infective material should not be used. These outlet traps were designed to offer less back pressure than the filters, hence more air can be passed through the unit without inflating the gloves. The preference in this work has been for an outlet filter.

It is important to use a clean air supply for ventilating the isolator. This may be accomplished by cleaning the air in a series of steps (FIGURE 12). The raw outside air is first passed through "roughing out" filters (commercial glass-fiber filters), then to a blower. Compressors that depend upon oil seals should be avoided. The air from the blower is conditioned, that is, brought to a controlled humidity and temperature by a series of heat exchangers and

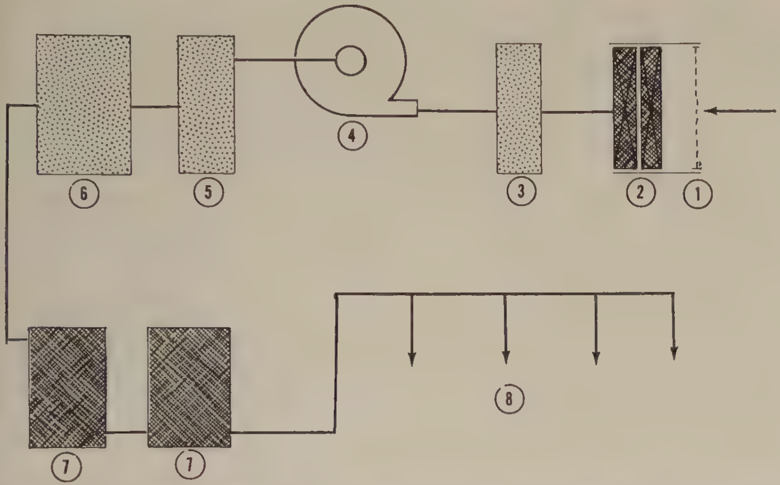

Figure 12. Schematic diagram of air supply to germfree isolators: (1) screen taking oarse particles and insects out of the system; (2) standard commercial glass fiber filters for oughing out the air supply; (3) preheater; (4) pressure blower (compressors with oil or water eals should be avoided); (5) exchanger for humidity control; (6) mixing plenum for tempera- ure and humidity control; (7) large glass fiber filters that can be sterilized and dried *in situ* these are in pairs to permit one or the other to be sterilized without interfering with the peration); (8) manifold to germfree isolator filters.

refrigeration, after which it may be passed through another series of filters, hence to the filter mounted on the germfree isolator. If the secondary filters are used, they should be in duplicate and capable of being sterilized and dried *in situ.* The secondary filters have not been used in the work reported, since tests of the air at the germfree isolator outlet show it to be very low in microbial count.

The amount of air passed through a germfree isolator will vary with the size and kind of isolator, the number of animals, the resistance of the outlet filter or trap and, particularly, the manner in which the animals are housed within the isolator. For most of this work using RSU-300 or -400 isolators, 1.5 to 3.0 cubic feet per minute of air has been used. This air flow does not overly nflate the gloves.

Sterility Checks on Germfree Apparatus and Animals

No attempt is made to detail the tests used, since this subject has been dis- ussed elsewhere (Reyniers, *et al.*, 1946, 1949a, 1949b). The routine examina- ions have been primarily for bacteria, but from time to time in the course of he work described herein other tests have been made for protozoa, viruses, ungi, and parasites.

Cotton swabs in test tubes plugged with cotton are taken into the isolator hrough the supply lock. Samples of freshly expressed feces, waste materials, ater, and animal surfaces are gathered on the swabs, and these are removed rom the isolator. They are cultured in thioglycollate broth incubated at

25°, 37.5°, and 55° C. for 24, 48, and 170 hours. Microscopic examination is made from wet mounts of the incubated broth and from the fresh samples , and stained slides are also examined. If further culturing is necessary this is done from the microscopic tests.

From time to time (averaging monthly in Group I studies) an animal from each isolator is sacrificed in the germfree isolator, and cultures are made of the intestinal tract, blood, liver, spleen, lymph nodes, and other tissues. These cultures are subject to microscopic and biochemical examinations.

From time to time the animals are especially examined for protozoa and, by blind passages of lungs and other organs for viruses.

It should be remembered that experience has shown that, if contamination occurs in a germfree isolator, all animals in that isolator will be contaminated, which makes microbiological examination more certain. Moreover, there is no competition from other microbes and from the environment that includes the animal. Finally, the animal itself is an excellent culture medium.

Housing and Maintaining Germfree Animals

There are certain practical limits imposed by the technique as to the numbers of animals of any given species it is possible to house and maintain in a germfree isolator. This is not entirely a matter of space.

It should be pointed out that the position of the operator is fixed, and there is only a certain area that can be reached in which objects can be handled directly. If the same isolator contains two or more pairs of gloves arranged in opposite or aligned positions, the isolator can be made larger, but the area covered by each individual remains the same. The coverage for an individual can be increased if objects can be brought to his position by means of extension devices. In such instances the animals must be housed in movable cages. The number of animals that can be housed in a single germfree isolator is also restricted by the need for sterilizing everything brought into contact with the animals. Such limiting factors include the size of the supply lock, the storage space available, and the length of time food can be stored.

At present food can be stored at isolator temperatures for from ten to fourteen days after sterilization before its nutritional value deteriorates. Water and wood shavings are limited in amount only by storage space. Even if large quantities of food and water are sterilized and refrigerated in a separate germfree isolator, then passed by a supply lock transfer to an isolator containing animals, this will not entirely satisfy the demands because, in the operation of an isolator, there is a need for frequent passages. If large rooms are built to house many animals, the gain may not be commensurate with the difficulties of entry and maintenance and the loss of a large number of animals if contamination occurs.

The RSU-100, -200, and -300 isolators used in this study represent the maximum size that can be managed by one individual from a standing position in which the entire arm can be used. The isolator is 28 inches in diameter and 46 inches long, with a floor space of 24 inches by 46 inches. The floor space is limited by the fact that the floor must be below the supply lock inner door

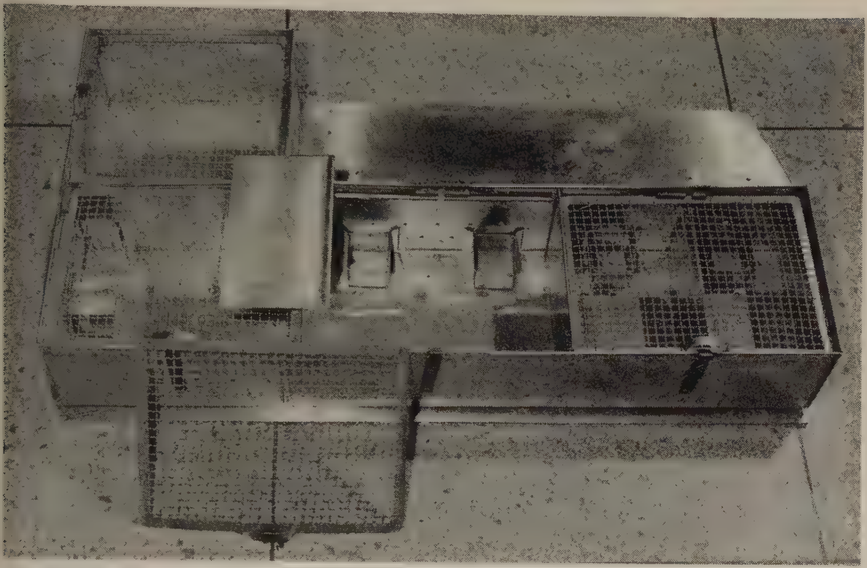

FIGURE 13. Mammal pens and floor for RSU-300 isolator. Removable partitions form compartments. Holes in side of pen furnish ventilation.

so that it can be opened. The supply lock is located on the midline, since experience has proved this to be best; in this position it is directly opposite to the operator. The space below the floor is used as a storage space for food, bedding, and other supplies.

Rats are housed in an L-shaped run (FIGURE 13), 44 inches wide and 7 inches deep, that can be divided by partitions into 3 or more compartments. Mice are housed in square glass jars 8 inches by 8 inches by 10 inches (FIGURE 14) fitted with screw-top screen lids. Chickens are housed in pens (FIGURE 15), 21 inches by 12 inches by 14 inches high. Guinea pigs are allowed the use of the entire floor space for one week after birth and are then caged in a rat run.

In a standard germfree isolator (RSU-300), it is possible to maintain 14 jars of mice, 5 adults to a jar (70 adults); 28 adult rats; 8 adult Bantam or 4 standard-breed chickens; or 10 adult guinea pigs. Unused floor space of 288 sq. in. minimum is necessary to permit manipulation of the supply lock door and general operations involved in reaching the subfloor storage space and stacking supplies. Because it is necessary to examine the animals through the window from above, it is inadvisable to stack the cages. This causes overcrowding and difficultly in working inside the isolator.

The RSU-400 isolator is 36 inches in diameter by 56 inches long, has a floor area of 34 inches by 54 inches, and is furnished with 2 pairs of gloves situated opposite each other. This isolator has been used only with mice because movable cages are necessary. In this isolator 25 mouse jars (125 adults) can be operated easily.

The REXU-3 isolator is 28 inches in diameter by 108 inches long, and is urnished with 3 pairs of gloves on the same side of the isolator. This unit

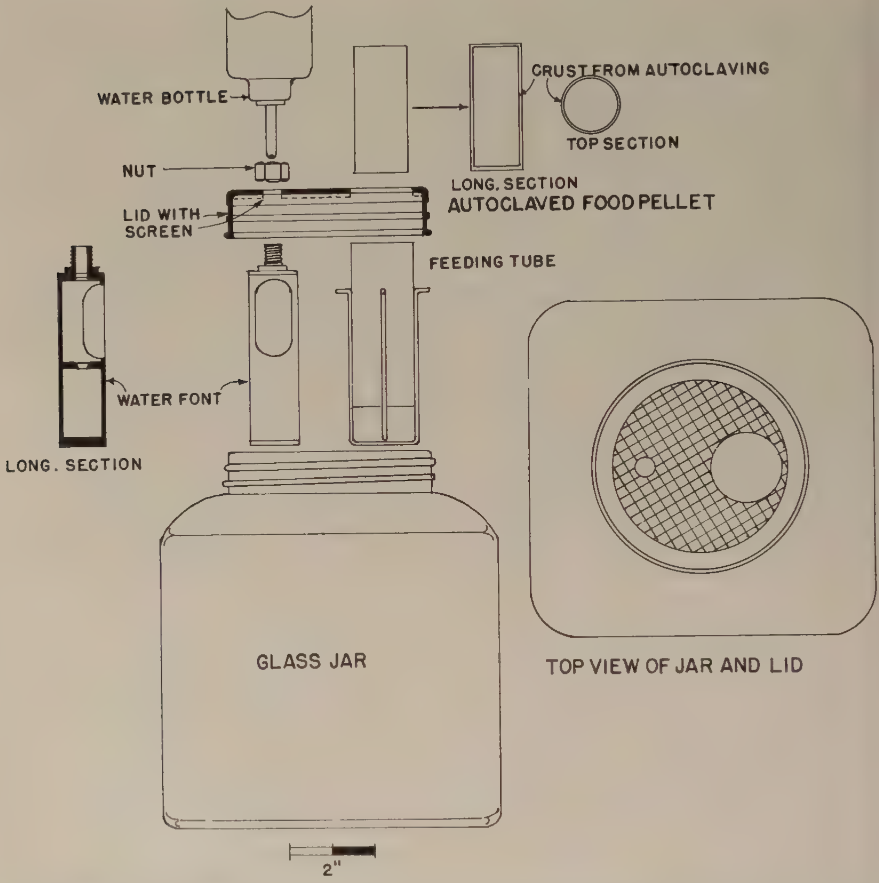

FIGURE 14. Schematic diagram of mouse jar, showing water font and feeding tube. On autoclaving, the compressed food pellet (2½ inches long and 1½ inches in diameter) forms a crust.

in which we have maintained chickens only, will easily house 16 Bantam or 8 standard-breed adults. Since the floor space is 26 inches by 106 inches, it can house as many as 34 mouse jars (170 adults) and approximately 50 adult rats. This isolator is equipped with 2 supply locks.

The RESU-1 isolator is 28 inches in diameter and 66 inches long, has a floor space of 26 inches by 64 inches and has been used only for guinea pigs in this study. Two pairs of gloves on opposite sides of the isolator and 4 supply locks permit a maximum of service. In this isolator, twelve adult guinea pigs can be maintained.

The amount of supplies that can be taken into a germfree isolator at one time is limited by the size of the supply lock. Since all standard supply locks are 9 inches in inside diameter and 15 inches long, it is possible to calculate

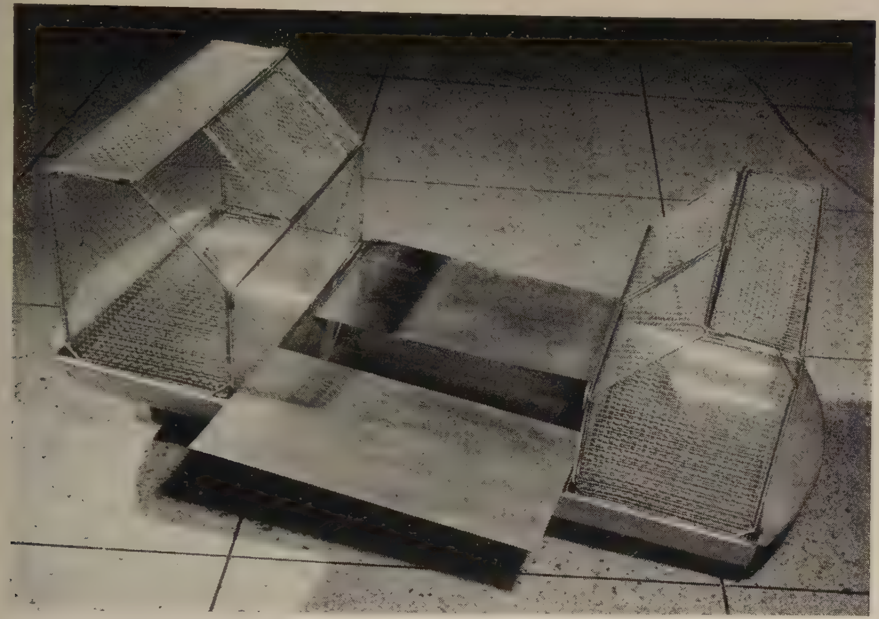

FIGURE 15. Chicken pens and floor for RSU-300 isolator. The center compartment is for storage of supplies, and the wire cages set over compartments to collect waste. These pens also can be used for guinea pigs and rabbits.

the number of passages necessary to maintain the species of animals housed in each type of germfree isolator. The size of the supply lock has been determined through experience as the most usable. Doors larger than 12 inches in outside diameter (the size needed for a standard supply lock of 9 inches inside diameter) are difficult to hold tight with a simple bar closure and take up too much floor space. When more supply-lock space is necessary, a second detachable lock can be fastened to the first, thus increasing the capacity without increasing the diameter. The accessory lock can be removed after use.

The solid food used for rats and mice is pelleted by compression into the Lobund pellet (Reyniers and Sacksteder, 1958), which is 1.5 inches in diameter and 2.5 inches long and weighs about 66 gm. (FIGURE 14, upper right). This pellet is made to be sterilized (autoclaved), at which time a crust forms around it so that it is not fragile and there is very little (about 2 per cent) waste when fed. The pellets may be fed from a tube to avoid soiling by the animals.

The water cans hold 400 ml. of water and are 3 inches in diameter and 4 inches long. Wood shavings are put up in packages of 120 gm., sufficient for one mouse jar, and are 4 inches by 8 inches by 2 inches thick.

A supply lock will hold at each loading as many as 12 cans of water (4800 ml.,) 60 pellets of food, (about 3900 gm.) or 12 packages of shavings (1450 gm.). The water and bedding are storable without deterioration, but the food should not be stored in the isolator for more than 2 weeks. Since other supplies must be taken into the isolator and, because any given passage may contain mixed supplies, the following average number of supply-lock passages per month have been made in the studies reported: RSU-300 isolators (mice), 12 passages; 8 passages for RSU-400 isolator (mice); 8 passages for the REXU-1 isolator (guinea pigs); 12 passages for REXU-3 isolator (Bantam chickens); 12 passages for RSU-300 isolator (rats); 8 passages for REXU-100 isolator (guinea pigs); and 18 passages for REXU-3 isolator (standard-breed chickens). In actual practice, which the figures given above represent, different experiments involving different species of different ages and numbers will demand a varying number of food autoclave passages per month. The numbers of rats, mice, chickens, and guinea pigs that can be maintained in the various isolators used in the study are summarized in TABLE 1, and the numbers of weaned rats and mice (21 to 25 days of age) that can be conservatively produced in an RSU-300 isolator are given in TABLE 2.

In operating germfree equipment, special attention has been paid to the proper care and use of the rubber gloves attached to an isolator. Each time an isolator is placed into operation, new gloves should be used; this is cheap

TABLE 1

OPTIMUM NUMBER OF ANIMALS MAINTAINED IN A STANDARD RSU-300 GERMFREE ISOLATOR*

Species	Average no. of adults per isolator†	Average no. of supply-lock passages per month‡
Rats	28	12
Mice	60–70	12
Bantam chickens	6–8	12
Standard chickens	4–6	14
Guinea pigs	8–10	8
Rabbits	2–3	8

* Floor space 24 inches by 46 inches.

† These numbers may be increased if younger and smaller animals are held, or by overcrowding by 20 per cent.

‡ An average of supply-lock passages would include food, water, bedding, instruments, and general and special supplies.

TABLE 2

NUMBER OF WEANED (21- TO 25-DAY-OLD) RATS AND MICE PRODUCED PER YEAR PER GERMFREE ISOLATOR RSU-300*

Species	No. of breeding adults/isolator		Average no. of young/litter	Percentages weaned 21–25 days	No. young weaned/ isolator/year
	Males	Females			
Rats	4	6	7.4†	80‡	240–250§
Mice	6	15	8.2†	80‡	320–340§

* Dimensions: 28 inches in diameter by 46 inches long.

† May be increased by selective breeding.

‡ May be increased by selection.

§ These figures are based on the removal of the young from the isolator at weaning and the maintenance of vigorous breeding stock.

insurance for an experiment. Each time the gloves are used and after each sterilization they should be washed, dried, and powdered. Careful inspection and reinforcement of rough spots are also necessary. Before an operator dons the gloves, his arms are covered with stockinette or he puts on a clean gown, and his hands are covered with clean, close-fitting cotton gloves. All operations inside the isolator that involve opening or closing the supply lock door or manipulating the animal cages are done only after putting on a pair of cotton work gloves over the rubber-covered hands. Using these techniques we have had very little trouble with punctured gloves. Furthermore, all mice are handled with long forceps with rubber-covered tips. Larger animals are handled with the work gloves already mentioned. Only in those instances where dissection of an animal or procedures demanding more sensitive touch are involved have the rubber gloves been uncovered. Finally, as a matter of good practice, access to the isolator should be limited to a minimum number of individuals. This is more important than the number of times the gloves are entered.

Man Hours Involved in Operations

It is extremely difficult to prescribe the man hours necessary for operating germfree equipment, because this depends to a certain extent upon its use. If the purpose is merely the rearing of animals, this is one thing but, in the studies reported, the purpose was experimental, requiring daily observation of each animal. In the Group I studies the animals were bred to a specific pattern. However, time approximations can be given for some operations.

In my experience it is better to assign a number of germfree isolators, together with the necessary help to operate the equipment, to a single investigator. In the Group I studies the equipment was operated by one technician, who has been able to maintain the necessary operations for ten isolators and to give limited assistance in the task of feeding. In this instance the individual concerned was responsible for disassembling an isolator, cleaning, testing, and sterilizing it, making all isolator-to-isolator transfers, all supply-lock passages, and other necessary maintenance. The food, water, and supplies came from central services and were packaged when received.

In Group II studies the maintenance operations were performed from central services. However, the equipment was under the direction of a single investigator.

Many of the operations necessary to maintain germfree equipment are performed seldom; others, such as feeding and caring for the animals, are done more frequently. The fact that a germfree isolator may be run for as long as 2 years before the animals are changed and the isolator disassembled spreads the actual hours of labor. Also, several isolators may be operated at the same time; therefore each would require less individual attention. The same thing may be said of supply-lock operations. These are factors that must be taken into account in any estimate of the time required to operate germfree equipment. Nevertheless, as a matter of information, the man-hours necessary in the various steps involved in the operation of the equipment are summarized in TABLE 3.

TABLE 3

APPROXIMATE MAN-HOURS NECESSARY FOR THE OPERATION OF GERMFREE ISOLATORS

Operations	Man-hours*
Disassembling and cleaning a germfree isolator	2
Assembling and testing for leaks (presterilization)	2
Initial sterilization	2
Supply-lock passage steam sterilized and vacuum dried	2
Isolator-to-isolator transfer via supply locks, including testing	2
Germicidal passage via liquid trap into isolator	2

* These times are average for the operations described in this report and are ample for careful work. Time can be cut by duplicating some operations such as, food autoclave passage or by taking short cuts if the attendant risk of contamination is accepted.

Cost Factors

The cost of working with germfree animals is governed by the apparatus, the installation, and the operations necessary to maintenance. The same can be said for any experimental work requiring specialized apparatus and techniques. If the purpose is to produce germfree animals as well as to use them experimentally, a different installation is necessary than if it were intended to use animals produced elsewhere. By the same token, if the interest in germfree life is continuous rather than casual, this will also influence cost. The point is that the cost of installation will depend upon the extent of the anticipated work. The cost of operation will remain about the same per isolator per day, regardless of the kind of apparatus used. In any case the situation is changing: it is entirely feasible today to produce animals (rats and mice) from breeder stock obtained from a central source and to continue production in a few germfree isolators set aside for the purpose. This is an added convenience as compared to complete dependence on a central service for all experimental animals.

The cost of metal germfree apparatus lies largely in the fabrication, which includes welding, machining of parts, assembly, grinding, and polishing. Fabrication costs are about the same regardless of the thickness of metal used in the shell. From a manufacturing point of view the use of light, (16-gauge) metal adds to the fabrication costs. From the point of view of service life there is no advantage to a lighter shell, as can be attested in our experience with the 16-gauge REXU-1 series of isolators, all of which collapsed after several years of use and required reinforcement with heavy bands. Since all isolators are mounted on rubber tires and ball-bearing wheels, the weight of the isolator is not important. Again, the best metal in our experience has been stainless steel as far as service life and maintenance costs are concerned. The saving in initial costs made possible by using mild steel is more than offset by the increased costs of maintenance and dependable function. Finally, the difference in costs between an unfinished (that is, with unpolished and unground welds) stainless steel isolator and a finished isolator is relatively small, and may not be worth the difference in view of the factor of ease of maintenance. The cylindrical shape for pressure apparatus is probably the best and is certainly the cheapest to machine and fabricate as compared to boxes and semiround shapes, but this is a matter of choice.

The Reyniers germfree isolators have been stripped down to the barest essentials consistent with the work for which they are intended. It is probable that, in time, manufacturing costs will be cut with economy in details of construction and with volume, but the changes in the basic isolator will not be great. Consequently isolators purchased today will function, like autoclaves, for years to come, and new isolators will be essentially the same. The cost of the present isolators may be written off over an anticipated twenty-five years. Changes that may be expected will be in size and kinds of accessories, but accessories may be added to any isolator. Isolators highly specialized to certain purposes have been and will be built. At present the cost of a standard germfree isolator is about the same as that of an autoclave.

The use of steam, heat, and chemical, or cold disinfection is a matter of choice. Both steam and chemicals can be used in operating metal isolators. Autoclaving food and supplies will probably continue to be the method of choice because it is safest. This being the case, permanently attached supply locks are necessary. If chemical disinfection is used in the initial sterilization of germfree isolator in an effort to utilize materials other than metal (Trexler and Reynolds, 1957), this again is a matter of choice. However, regardless of the method used to produce sterilization, the essential operations involved—for example, in supply-lock passage—remain the same for any equipment.

Where initial costs of apparatus are important, as may be the case with investigators who have a transient interest in germfree life and who depend upon receiving germfree animals from a production center, there are many devices useful to the purpose. These range from isolators to the so-called "dry box," in the construction of which a variety of materials have been used. Such equipment is useful for short-term experiments, for example, hemorrhagic shock, so that it has its place in the scheme of things. For the investigator, the problem lies in a decision as to the extent of the work and the kind of experiment undertaken. As already pointed out (Reyniers, 1957), there are many devices that can be constructed in the laboratory in which limited work with germfree animals can be done; accordingly, there is no reason why anyone interested in this field of work cannot enter it.

The costs of producing germfree animals has declined steadily with improved apparatus and better routine methods. The cost to the investigator has been minimized by the fact that breeding stock may be shipped from a production center to the investigator and a local colony instituted. Apart from the initial cost of apparatus, which is comparable to initial costs of setting up any specialized laboratory, the cost of maintaining a colony of germfree animals compares well with the costs of producing disease-free or "clean" animals. Researchwise, there is no way of comparing the costs of using germfree animals with those of using conventional animals, since it must be presumed that conventional animals will not answer the purpose or they would be so used. Since there are, at present, no commercial centers for rearing and shipping germfree animals, any costs would have to be estimated on an experimental basis, just as the costs of antibiotics in the initial stages of development could be estimated but had no real basis until these products were produced commercially.

Finally, the initial costs of setting up a germfree installation will vary according to the kind and amount of work to be done. In some instances, one or two isolators may satisfy the needs; in other cases more isolators will be necessary. If cesarean sections are to be performed and the animals reared by hand-feeding, specialized isolators are necessary. This seems obvious. At any rate, on the basis of the information given, some estimate can be reached concerning the numbers and kinds of germfree isolators necessary. The costs of these isolators must be added to that of a proper air system and quarters, as in the case of any germfree system.

Maximum Time a Germfree Isolator Can Be Operated Continuously

In the past it has been a matter of routine to remove animals from one to another isolator about every 6 months. In the Group I studies this routine has been lengthened to 10 months. However, in the interests of determining the maximum time that a single isolator can be maintained before contamination in routine operations, the RSU-400 isolator was selected for test. This isolator is of mild steel, with a baked enamel finish, and is 36 inches in diameter and 56 inches long. It is equipped with standard Trexler Air Filters on inlet and outlet, and has two pairs of gloves and one standard supply lock.

This isolator was set into operation on August 8, 1956, with 21 mouse jars and a full complement of mice. It has been maintained without changing the animals to date (May 1958), and is still in operation. During this period more than 180 supply-lock entrys have been made. The animals and isolator have been maintained constantly and have tested free from contamination.

Such information is of considerable practical value, since it demonstrates that fewer isolators are necessary if their dependability is increased. By the same token, less time is needed to maintain the animals, and there is less risk in transfer to another germfree isolator. Finally, a greater sense of security and dependability may be expected with the use of steel equipment maintained by steam sterilization.

Number of Germfree Isolators Needed

The number of germfree isolators necessary to an installation will depend upon the kind of work for which they are used. There are instances in which one isolator may suffice if germfree animals can be produced commercially and shipped to an installation. If, on the other hand, the experiments are of long range (several months to several years) and greater numbers of animals are required, more germfree isolators will be necessary. To enable the investigator to estimate his needs, the following information should be useful.

A germfree isolator such as the RSU-300 (28 inches in diameter and 46 inches long) occupies a floor space of 24 inches by 46 inches, and will accommodate 12 mouse jars, or approximately 60 adult mice. In it a pen arrangement may be used for rats (FIGURE 13), which will accommodate 16 to 20 adults. The same isolator with 2 chicken pens (FIGURE 15) will accommodate 4 adult chickens or 8 adult Bantams.

With the RSU-300 isolator used only for breeding animals, from which the young are removed at weaning to other isolators, it is possible to rear the following numbers of germfree mice and rats.

Rats. Assuming 6 breeding females and 4 adult males per isolator with a litter size of 7.4 and about 80 per cent weaning, approximately 240 to 250 weaned animals can be produced per year.

Mice. Assuming 15 breeding C3H females and 6 adult males per isolator with an average litter size of 8.2 and about 80 per cent weaning, approximately 320 to 340 animals can be reared per year. These figures are approximately the same for the Swiss mice.

It is possible, of course, to obtain more young from a single RSU-300 isolator if greater care is taken to select the best breeding stock and by putting more animals into the isolators. While we have not been especially interested in rearing a maximum of weanling animals, we have found that the above numbers can be obtained, as well as maintained, with a single isolator.

The figures given for the number of adult animals that can be held in a germfree isolator can be increased if younger animals are used. The problem is not only the maximum number that can be housed in a single germfree isolator, but also the number that can be supplied with food, water, and bedding, since these supplies must be sterilized and also stored until used. With the larger isolators, proportionally greater numbers of young can be produced and brought to weaning.

It is not possible to state exactly the number of isolators necessary to an installation without knowing the kind and extent of the operation. If the intention is to perform cesarotomies and to hand-rear the young, it will be necessary to have an ROPU-200 isolator with one or more RSU-300 isolators in which hand feeding can take place. If chickens are to be used it will be necessary to have germicidal traps and incubation isolators, both of which are accessories. There are other accessories, such as special interior equipment, deep-freeze units, vacuum sweepers, external water sterilizing tanks, end-to-end connecting links, homogenizers, filtering units, and accessory supply locks.

A complete installation has been variously estimated (Reyniers, 1957) as: 1 ROPU-200, 1 REXU-200, 10 RSU-300, 1 RGTRPO with RIX-200 incubator, and 1 accessory supply lock, with the necessary interior equipment, such as animal pens. This installation permits work with chickens and small mammals that are obtained by cesarotomy, hand-reared, and produced as a colony, together with 8 to 10 isolators for experimental purposes. However, as has been pointed out, it is difficult to determine the needs of individual groups of scientists; more isolators may be added as needed.

RESULTS

Germfree Apparatus Used in this Study

Two groups of Reyniers germfree isolators, respectively designated as Group I and Group II, have been used in the results here reported. This equipment

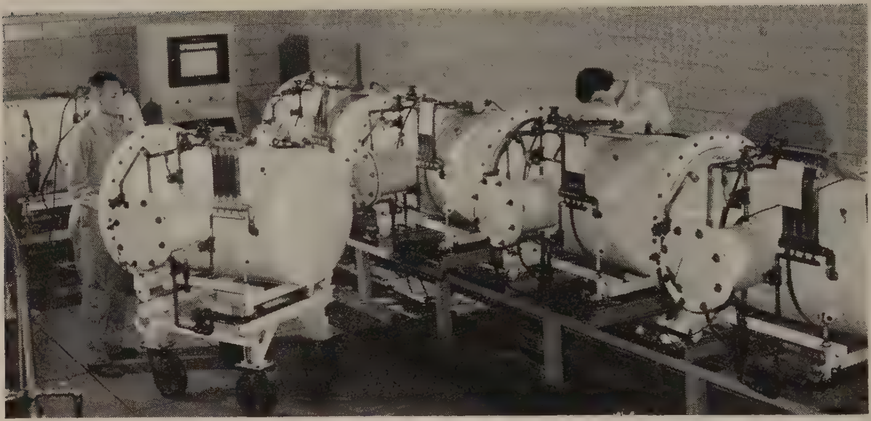

FIGURE 16. Types of isolators used in these studies. The RSU-200 isolators are mounted on a rack. They are moved about on the truck as shown.

was made in the Lobund Institute shops and must be considered developmental. No attempt was made to polish the welds or to finish this equipment completely. Some of it is made of stainless-clad steel; some of mild steel, with painted surfaces.

Group I apparatus. This consists of the following germfree apparatus: 5 RSU-300 rearing isolators; 1 RSU-400 isolator; 1 REXU-3 isolator attached to the end of the large-scale colony apparatus (this is a three-man isolator); 2 RSU-100 isolators and, from time to time, 1 or 2 RSU-200 isolators. The apparatus is shown in FIGURE 16. The RSU-200, -400, and the REXU-3 isolators are mild steel, painted inside and outside; the RSU-100 and 300 isolators are stainless-clad steel.

The ventilation systems vary somewhat in design on some of the isolators, but the air filters are all of glass fiber. On a few isolators a germicidal trap has been used on the outlet; the rest of the isolators are equipped with standard Trexler outlet filters. The series 100 isolators (stainless steel) were placed in operation in 1950; the series 200 isolators (mild steel) in 1950; the series 300 (stainless steel) in 1953; the series 400 isolator (mild steel) in 1955; and the REXU-3 isolator (mild steel) in 1950. These isolators have been used continuously since these dates.

All germfree isolators except the RSU-400 are operated from racks; the latter is operated from its own stand. This means that the isolators on racks are operated from a central control panel, while those on stands are operated from a sterilization cart. Experience has shown that the latter method is more certain, since long supply lines are difficult to maintain. Also, by operating from a sterilization cart, the number of valves on the germfree isolator may be cut to a minimum since they need not be duplicated on each isolator inasmuch as they are on the cart.

All doors, windows, and sterile locks are sealed against rubber gaskets. The end door gaskets may be changed only during the disassembly, but those on the supply lock may be changed at any time. Since this is a critical area, such changes are more frequent. The Saunders-type valve is used almost exclusively on this equipment. The diaphragms are changed on inspection when necessary.

Group II apparatus. These germfree isolators were used exclusively for rearing guinea pigs and for using these animals in studies on amebiasis (Phillips *et al.*, 1955). The following kinds and numbers of germfree isolators were used in these studies over a 5-year period: one REXU-1 isolator (stainless steel) equipped with 2 pairs of gloves and 4 supply locks, and 3 series RSU-200 isolators (mild steel). The RSU-200 isolators are of the basic design already described. The REXU-1 isolator is on its own stand, but is fixed in position; the RSU-200 isolators are on racks (FIGURE 16). The REXU-1 isolator was commercially made of stainless steel with polished welds and finishes. It was made of light-gauge (16-gauge) metal and collapsed after several years of use. It was necessary to reinforce it with heavy metal bands welded into place. This isolator was placed in operation in 1946 and the RSU-200 isolator in 1950.

One other isolator, the ROPU-2 operating unit (mild steel) has been used. It was placed in operation in 1950 and is still in use on the average of twice weekly.

Group I Operations: March 1955 to October 1957

This operation has been concerned with cancer studies in germfree C3H and Swiss mice, white rats, and Bantam and standard Leghorn chickens. Because of the nature of the study it has been necessary to breed the mammals and to hold some of them for their life span as well as to conduct experiments of shorter range. For this reason only the mammal operation is considered. In this operation within the period of 31 months there has been one contamination in an RSU-200 isolator containing C3H mice. This was probably due to a warped outer supply-lock door that leaked during sterilization and became contaminated when a vacuum was created in the lock.

A résumé of the operation is given in TABLE 4. All the isolators involved were operated by Robert Sieczko.

In an effort to determine how long a germfree isolator could be operated without changing the animals, the RSU-400 isolator was placed in operation with a full complement of C3H mice on July 6, 1955, and was still in operation without contamination on March 4, 1958. During that period there were more than 180 supply-lock passages.

The oldest C3H mice are 900 days plus, the Swiss mice are 900 days, and the rats 600 days plus. We have had no occasion to hold chickens (they were not bred and were not held) more than 12 months, and have had this phase of the work under our direct supervision since May 1957. During that time 60 chickens have been hatched in 5 experiments. We had no contaminations in this short run.

There have been no failures due to glove breaks, through the air filters, or because of failure of the apparatus, except as noted.

TABLE 4

GROUP I OPERATIONS OF GERMFREE ISOLATORS WITH C3H MICE, SWISS MICE, AND RATS

(March 1955 to October 1957)

Total number of germfree isolators used	6
Total number of C3H mice reared and held	375
Number of germfree isolators used for C3H mice	3
Total number of germfree Swiss mice reared and held	327‡
Number of germfree isolators used for Swiss mice	2
Total number of albino rats reared and held	65‡
Number of germfree isolators used for albino rats	1
Total number of supply lock passages	1210
Total number of isolator to isolator passages	64
Total number of initial isolator sterilizations	32
Total number of contaminations due to apparatus failure*	1
Oldest animals in days and species held in the isolators	600 albino rats 900 C3H mice 900 Swiss mice
Number of generations from start of operations†	4 albino rats 8 C3H mice 10 Swiss mice

* This is the only failure in these experiments and was due to a warped outer supply lock door which failed when a vacuum was created in the lock following sterilization.

† This period is from 1953.

‡ The figures given for numbers and kinds of animals are not production numbers and must not be confused with the capacity of these isolators to produce young. These are adult animals that breed and rear their young in the isolators and are held for their life span.

Group II Operations: August 1952 to November 1957

This work was conducted at Lobund Institute by Bruce P. Phillips of the National Institutes of Health, Bethesda, Md., and has been confined to rearing germfree guinea pigs for use in studies on amoebiasis. Since this has been largely an experimental study and involves cesarean operations, the results are of interest and supplement those of Group I operations.

Three germfree isolators were used in this operation, one REXU-1 examining isolator and two RSU-200 isolators. The ROPU-2 isolator was used for the cesarotomies. The results of this study are shown in TABLE 5. The operation of this equipment was performed by the service division of Lobund Institute, and over that period of time 5 individuals were involved in the operations. However, these isolators were under the direct supervision of Phillips, and all feeding and experimental work was performed by him or by a technician under his direction. The cesarean operations were largely performed by technicians from the service division of the Institute.

Group III Operations: June 1957 to April 1958

The work done by this group was confined to chickens using RSU-100, -200, and -300 isolators operated for cancer studies. Successive experiments are reported. The results of these studies are shown in TABLE 6.

The total number of isolators used for chickens never exceeded 4 in the studies reported. The number of days for each experiment was dictated by the demands of the experiment. The single contamination occurred because of

TABLE 5

GROUP II OPERATIONS OF GERMFREE ISOLATORS WITH GERMFREE GUINEA PIGS*
(August 1952 to November 1957)

Total cesarotomies	298†
Total number guinea pigs from cesarotomies	963
Total number of experiments	114
Total number of experiments germfree	98
Total number of experiments contaminated	16
Causes of contamination:	
Failure due to surgery	1
Failure due to intended marginal sterilization of new experimental dietary regimes but not to apparatus	11
Failure of apparatus (gloves)	2
Failure due to unknown causes	1
Failure due to contaminated amoeba	1
Total number of times the germfree operating isolator (ROPU-2) was used	115
Total number of initial isolator sterilizations	230
Approximate total number of supply lock passages	465
Approximate total number of germicidal trap passages	110
Age of oldest animals	14 months

* These results were made available by Bruce P. Phillips, National Institutes of Health' and are from work he did while stationed at the Lobund Institute. His experiments were with germfree guinea pigs and amoebiasis.

† Multiple cesarotomies into germfree isolator.

TABLE 6

OPERATION OF REYNIERS GERMFREE ISOLATORS (RSU-100, -200, -300) WITH CHICKENS*
(June 1957 to April 1958)

No. of experiment	No. eggs set	No. chicks hatched	Time (days) of experiment	Status of chicken	No. of lock sterilizations
1	15	15	180	GF†	4/mo.
2	12	10	79	GF	4/mo.
3	20	14	150	GF	4/mo.
4	20	12	127	GF	4/mo.
5	17	8	60	GF	4/mo.
6	15	11	92	GF	4/mo.
7	15	10	81	GF	4/mo.
8	15	4	5	Contam.‡	0
9	7	6	100	GF	4/mo.
10	15	14	170	GF	4/mo.
11	14	10	160	GF	4/mo.
Totals	180	128 (71%)	Av. 117 Days	10—GF 1—Contam.	160

* These experiments were concerned with induced tumorigenesis and this will explain the termination days of each experiment.

† Germfree.

‡ Contamination brought in with eggs. This was a poor hatch and poor quality of egg shells.

failure to sterilize the shells of a poor clutch of eggs. The poor quality of these eggs is further borne out by their low hatch in the germfree isolator. No failures were due to apparatus or during an experimental run.

Keeping chickens in germfree isolators makes it necessary to stretch a supplementary screen of surgical gauze over the outlet filter from inside the isolator. This filter must be changed daily because the dust and feathers shed by chickens will plug the glass fiber outlet unless the preliminary screen is used.

If an isolator is not overcrowded with chickens and if the watering devices are watched, no problems of moisture inside the isolator will be encountered at the normal rate of air flow. The numbers of chickens shown in TABLE 6 are young chickens and from single hatches. Multiple hatches in a single isolator should be avoided. Each hatch should be held separate until sufficiently tested for contamination (usually 21 days), at which time hatches may be combined if these are not contaminated.

Summary

Apparatus for rearing germfree animals has been standardized in design and construction to a point where it can be produced commercially, thus making it available to investigators. The operation of this apparatus and the techniques for rearing the animals can be reduced to dependable routines so that experimentation is possible. Because this is the present status, it is now possible to examine the operation and function of the apparatus with some degree of certainty.

Only the Reyniers germfree apparatus has been considered in this report, because it is the only apparatus that we have used and because it has been developed over a period of 25 years. Because the device has become standardized in design and use an analysis of its operations becomes meaningful.

The basic design of this apparatus is considered. Essentially, it consists of a series of isolators that can be connected for transferring germfree animals and carrying them through generations without contamination. The animals can be held through their complete life span. This apparatus is made to be sterilized *in situ* with steam under pressure and, while it may be used with chemical sterilization, the preferred method is heat. Because the device is made to be used with steam pressures, it is strongly constructed and will stand continual use for years.

Two groups of apparatus, sufficiently standardized for the purpose, are considered. Each group was directly under the supervision of a single investigator. Group I consisted of 6 germfree isolators operated by a single technician (a single technician can properly operate 10 isolators). This group of isolators was used for cancer studies, primarily with mice and rats. Mice were bred to the tenth generation, as were rats, and held for more than 900 days. Group II studies involved only guinea pigs, and included the recording of cesarotomies, rearing, holding, and experimenting on the animals. The two experimental groups cover the essential operations necessary to obtaining, rearing, and using germfree animals.

The eight basic operations involved in rearing germfree animals are: (1) disassembling, assembling, and testing the germfree isolator for leaks; (2) initial sterilization of the isolator; (3) placing germfree animals in the isolator; (4) supplying the animals with sterilized food, water, bedding, and necessary instruments through supply-lock operations; (5) passage of heat-labile materials and embryonated eggs into the germfree isolator; (6) sterility checks on the

animals and isolator interior; (7) sterilization of air for ventilation; and (8) housing and maintaining the animals.

The results of these studies are discussed. From the results it is possible to conclude that a germfree isolator may be operated without contamination for at least 21 months continuously. Rats and mice may be reared germfree for at least 10 generations and for as long as 900 days. The operations are no more expensive in terms of manpower or time than with any kind of germfree apparatus. In the Group I operations one contamination occurred due to a warped outer supply lock in more than 2 years of continuous service, involving over 1200 supply-lock passages, 64 isolator-to-isolator transfers, and more than 700 animals. In the Group II operations 114 experiments resulted in 16 contaminations: 11 due to experiments involving marginal sterilization of new type diets; 1 because of surgery; 2 due to apparatus failure (punctured gloves); 1 to a contaminated inoculum; and 1 to causes unknown. Over 900 guinea pigs were used in 5 years of operations, and some animals have been held for 14 months. These studies were performed with apparatus made in the institute shops and must be regarded as developmental.

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HOUSING OF DISEASE-FREE VERTEBRATES

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Introduction

Let us begin by differentiating between germfree and disease-free vertebrates. The germfree animal is free of all microorganisms, whereas the disease-free animal may be described as one free of pathogens and the clinical signs that they produce. Actually, as of this writing, common agreement has not been reached on terminology for the latter type of animal. Suggested definitions are: "animals free of commonly occurring pathogens and parasites," "specific pathogen-free," "pathogen-free," "cesarean-derived," and "disease-free" animals. Although we refer to these rats in our laboratories as CDs (cesarean-derived) for the sake of convenience, we shall refer to them in this paper as disease-free.

I have surveyed the limited field of existing commercial and noncommercial producers of disease-free vertebrates and have pooled the findings; however, most of the detailed information and illustrations are from our own institution. At present the different methods of housing such animals are not the results of controlled laboratory experiments, but it is hoped that in the future a reference laboratory will be maintained to check the validity of our currently accepted practices.

Floor plans. The first consideration in housing disease-free animals is a practical working floor plan. Since at this time the commercial production of these animals is devoted principally to rats, the techniques described concern that species, but can probably be extrapolated to other species.

Today we use three basic floor plans. Plan 1 is the single-man animal quarters in which one caretaker works alone, Plan 2 is a compromise in which two individuals work in one area, and Plan 3 utilizes large areas in which two or more people work. Some of the advantages and disadvatages of each of these plans are considered below.

In Plan 1, the single small room minimizes the introduction of contaminants by personnel, and a single individual holds full responsibility for achievements or failures. In some instances, however, it has been found that caretakers dislike working alone.

Our Plan 2 breeding rooms are operated by two caretakers. After considering personnel absences and vacation periods, at which time a second person must inevitably enter the breeding rooms, regular use of two equally trained people provides a reasonable compromise.

Plan 3 provides a single large breeding facility and a similarly large holding area, each manned by perhaps six or seven people. Apparently this system has proved successful for many years.

The proponents of Plan 1 feel that the added personnel in the other plans increases the risk of introducing contamination, whereas the users of Plans 2 and 3 feel that, with a tight barrier, more efficiency can be obtained with two or more individuals. Time and more controlled experimentation may answer this question.

Building goals. It is generally accepted that a disease-free colony building must have as many of the following goals as possible incorporated in its plans.

Since wild rodents and vermin are carriers of so many varied infections, the building must be rodent and vermin proof.[1] A windowless building with a minimum number of doors is an integral part of rodent and vermin control. A fire-resistant structure not only is good insurance, but would necessarily be made of rodent- and vermin-proof materials. A vapor barrier enables economical control of humidification in winter and dehumidification in summer.[2] Impervious smooth interior finish enables a high degree of cleanliness to be maintained. Air-conditioning for controlled environment is extremely important and will be taken up separately.

Basic construction. To accomplish the building goals set forth we have incorporated the following features in our construction. The foundation is 12-inch reinforced concrete. The side walls are 12-inch cinder block, finished on the inside with an adhesive vapor seal to which is applied 2 inches of Foamglas* rigid insulation.[2] The finish is hard, smooth plaster covered with a scrub-resistant paint.[2]

The 40-inch clear-span steel bar joists free us of interior posts. The steel roof decking is affixed above the bar joists. Foamglas insulation is applied to the steel and finished with a 5-ply tar-and-gravel-finished roof. The use of wood is kept to a minimum. The only window of the building is in the staff dining area and is of Thermopane† construction. All floors are at least 12 inches above grade to eliminate dampness and are composed of 5-inch reinforced concrete, painted with an abrasion-resistant plastic paint.[2] The ceilings are constructed of corrugated aluminum, which requires no care and is rodent and vermin proof and fire resistant.

Air conditioning. This subject includes ventilation, heating, cooling, humidification, dehumidification, and filtration of air. Before entering the main system all outside air should be filtered first for large particles with standard dust-stop filters, and then for microscopic particles with secondary filters. Removal or destruction of bacteria can be accomplished by electrostatic precipitators, charcoal filters, throwaway or washable filters, or ultraviolet batteries in the ducts. All of these have been used with apparent success. We filter out particles as small as 0.3 μ with Aerosolve‡ 85 filters.[2]

Three different approaches to air conditioning and ventilation are being employed today. One method utilizes one main unit for the entire breeding and holding facilities, a second uses smaller independent units for each room, and the third employs one system for the breeding rooms and an independent system for the holding area. This shows an obvious lack of unified thinking, but each system is empirically sound.

Humidification is accomplished by admitting controlled amounts of steam or atomized hot water into the air stream. A relative humidity of 50 to 60 per cent is most satisfactory. If the air change is minimal, humidification can also be accomplished by mechanical humidifiers suspended in the area. Maximum air diffusion is accomplished by many outlets with multiveined diffusers. Drafts are a much more serious factor in a conventional colony where airborne bacteria counts are high; however, it is generally considered good practice to minimize drafts in all animal colonies. Cooling and dehumidification are accomplished most efficiently by a refrigeration system. With controls in each area, even temperatures, whether high or low, are ensured by an alarm system.

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* Product of Pittsburgh Corning Corp., Pittsburgh, Pa.
† Product of Libbey-Owens-Ford Glass Co., New York, N. Y.
‡ Product of Cambridge Filter Company, Syracuse, N. Y.

Barrier system. The most desirable floor arrangement and basic structure are of little value unless a rigid barrier is maintained around the animal rooms. This barrier permits the entry only of materials that have been subjected to some form of sterilization. It is generally agreed that the barrier system is the most practical approach to the mass production of disease-free rats. Once a colony area has been sterilized and a known clean nucleus admitted, the barrier system commences to operate. Everything subsequently entering the building must be sterilized. Personnel probably present the greatest single hazard involving the introduction of contamination. In our laboratories the following procedure is rigidly followed. The outside door has a 4-inch raised threshold with complete rubber gasketing to ensure tight closure. Its outer surface is covered with electric fly screens to prevent insects from resting on the door and subsequently entering with personnel. The outside door is electrically interlocked with the next three doors so that no two doors can remain open simultaneously. As long as the outer door remains open an insecticidal aerosol fogs the entry lock.

When the outer door is closed, personnel enter a pressure lock in which outer clothing and shoes are left, then proceed in stocking feet and undergarments to a clean area, where they scrub up prior to putting on sterilized outer garments. This clothing consists of plastic boots, operating room gown or laboratory coat, cap, and optional rubber gloves. From this point the person is within the barrier and can enter the clean-animal quarters and can travel freely within that particular area. Cross traffic between areas is prohibited.

Bedding. Animals can be raised on wire or can be housed directly on bedding. In either case, mothers with litters must be on some sort of sterilized bedding material.

Wood shavings compose the bedding most commonly used and can be autoclaved in small quantities, subjected to dry heat in an oven, or gas-sterilized with special equipment.

In our laboratories we are able to sterilize eight intact bales at one time with a large two-door ethylene oxide autoclave. Ethylene oxide has been used for many years for sterilizing spices[3] and, more recently, is being used in hospitals[4] and pharmaceutical houses to sterilize thermolabile materials. Its bactericidal activity has been well described.[5-8]

After the shavings are removed from the clean side of the Castle sterilizer,*

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* Product of Wilmot-Castle Company, Rochester, N. Y.

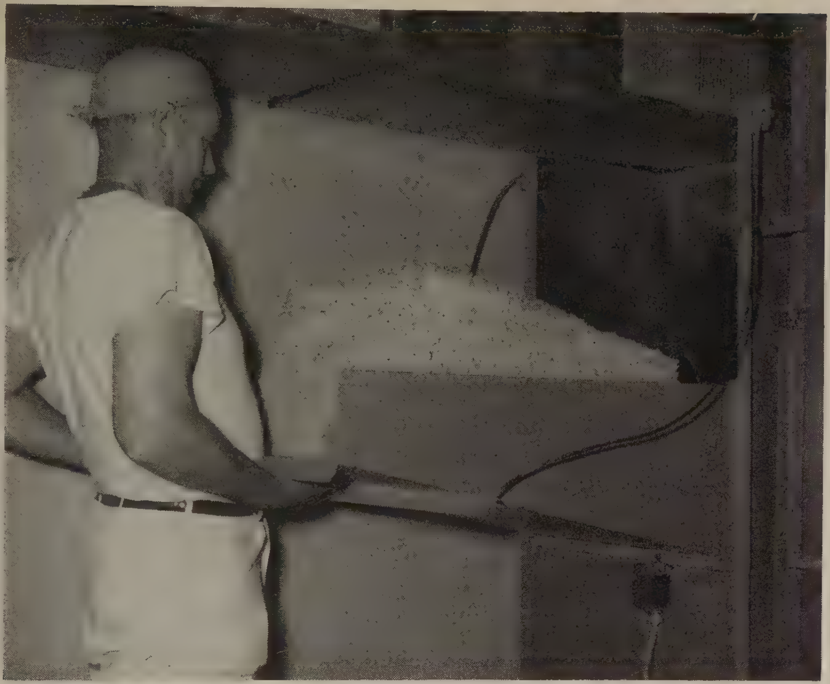

FIGURE 1. Wood shavings, used as bedding, being loaded into a bale breaker that feeds the material into bucket elevators that bring it to a belt network for distribution. These shavings are sterilized by ethylene oxide in the autoclave shown in FIGURE 2.

they are loaded into a bale breaker and then delivered into steel storage hoppers in the animal areas by bucket elevators and belt conveyors (FIGURE 1). The caretaker can then obtain sterilized bedding in each room by lifting the unloading gate on the hopper. Personnel traffic is greatly reduced by transporting the bedding in plastic-enclosed overhead belt conveyors, and only the caretaker in his particular area handles the bedding.

Feed. Feed can be formulated to withstand reduction in value by autoclaving and dry heat. However, ethylene oxide sterilization presents problems of initial destruction as well as continuing destruction of certain B vitamins and amino acids. This work is well described in the literature.[9, 10] We are using D & G[2] pasteurized rat diet* baked at 600° F. for 7 min. This food is received in high-density polyethylene bags that can withstand surface sterilization at 240° to 250° F. We load our dual-purpose autoclave with one-half-ton lots of this feed and subject the plastic bag to steam at 240° F. for 15 min. After removal in the clean area the food is dumped into a bucket elevator and distributed by belts in the same manner in which the bedding is handled (FIGURE 2).

Feed that has previously been sterilized also can be transported in galvanized

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* Product of Dietrich and Gambrill Company, Frederick, Md.

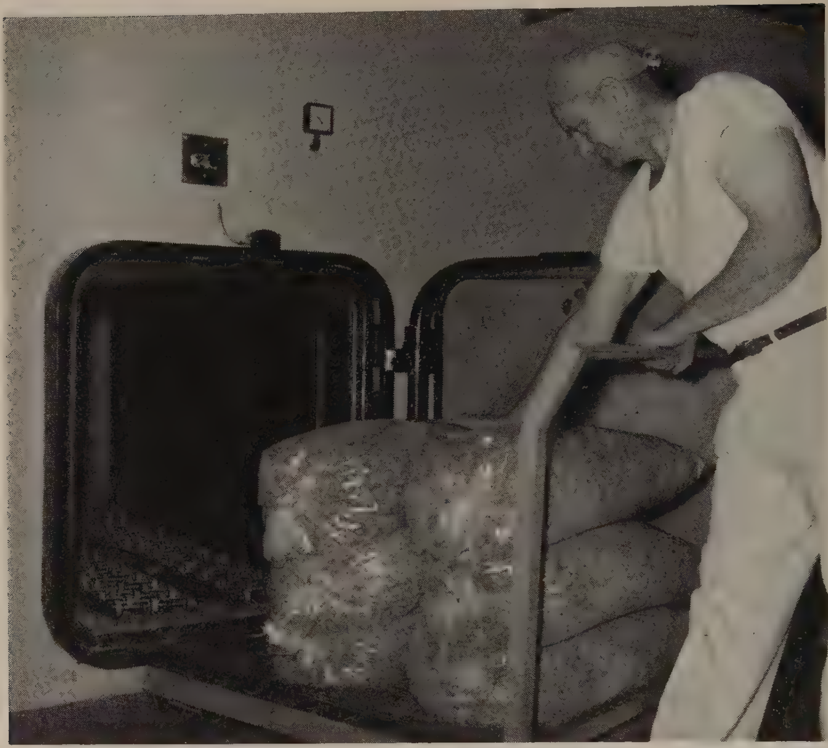

FIGURE 2. Previously pasteurized animal feed being removed from a combination gas and steam autoclave after the plastic bags have been surface-sterilized by steam.

iron cans or polyethylene bags and immersed in germicides or passed through low-pressure steam chests.

Removal of soiled bedding and excrement.[2] Consideration must be given to the removal of soiled bedding in such a manner as to eliminate the entry of wild rodents or other vermin. Bedding can be dumped into a floor pit whence it is taken outside of the building, by screw conveyors, or it can be removed in closed metal containers and passed outdoors through controlled exit hatches that are subsequently sprayed with insecticides. Although both of these methods have proved practicable, we felt that further insurance was needed and that labor costs might be controlled better if a central vacuum cleaning system were installed.

In 1952 we installed in our "conventional colony" building a pneumatic system for removing floor sweepings and for general housekeeping. During the next 4 years we ultimately cleaned animal cages directly with 25-foot vacuum hoses attached to overhead metal tubes.

In constructing our new colony building we embedded our main vacuum lines beneath the 5-inch concrete floors to conceal them and to eliminate noise. In the floor of each animal area, in the shipping rooms, in the grain and shav-

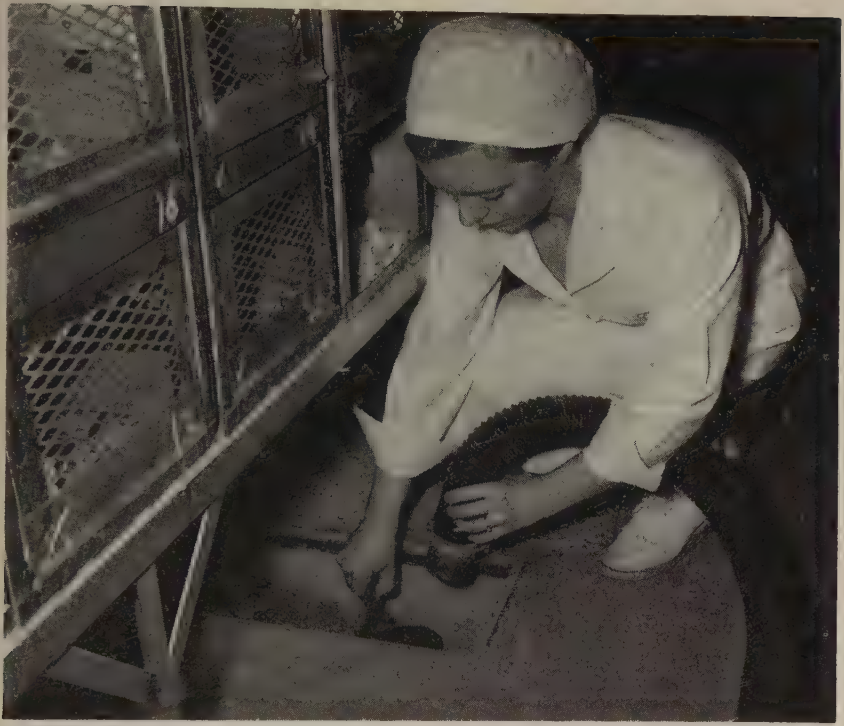

FIGURE 3. Animal technician plugging a 25-foot vacuum hose into a floor outlet. A pin in the center of the pressure cap prevents the hose from being blown loose.

ings room and in the equipment room are standard plug-in connections for vacuum hoses (FIGURE 3). Consequently, a caretaker in any part of the building can plug in a 25-foot hose, clean the cages (FIGURE 4) and, seconds later, safely deposit this bedding 120 feet from the building in a truck that is dumped several miles away. The system is designed to enable four people to perform their cleaning chores simultaneously. It is impossible for foreign material to re-enter the system, since it is under vacuum and with a rate of travel of 5000 feet per minute.

Watering. Within a tight barrier system, some have used an open water-pipe system with continuous flow, a pipe passing through each cage. After much research on automatic watering we felt that the individual-bottle system was the safest and most practicable. We therefore designed our cages to accommodate pint bottles with straight drinking tubes. Water outlets are in the floor in the same utility receptacle with the vacuum outlets. Regular heavy-duty air hoses with "plug-in" water connectors on one end are used. On the water end we have adapted commercially available restaurant fixtures so that the bottle may be pressed against a plate, releasing the water under pressure. Removal of the bottle from the fixture automatically stops the flow of water (FIGURE 5).

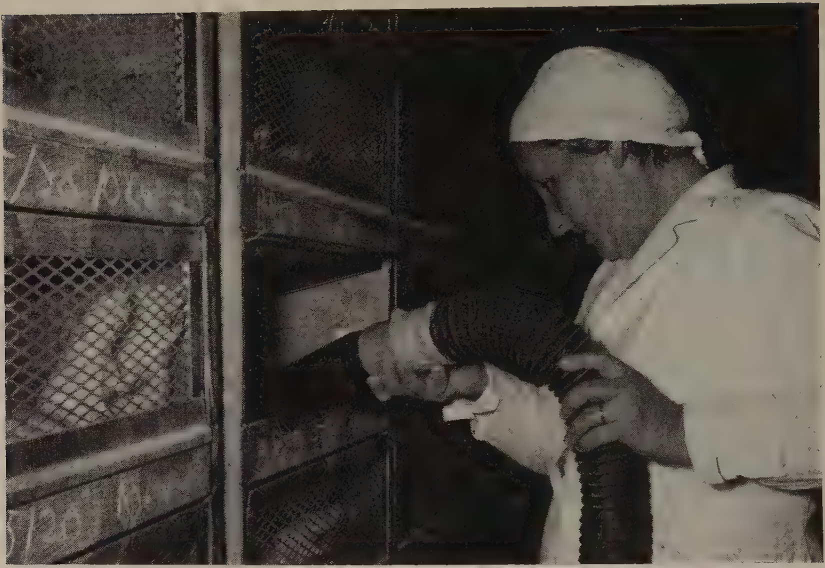

FIGURE 4. Animal technician cleaning a breeding cage with a specially designed vacuum nozzle. Note the information regarding the animals that is written in chalk on each cage.

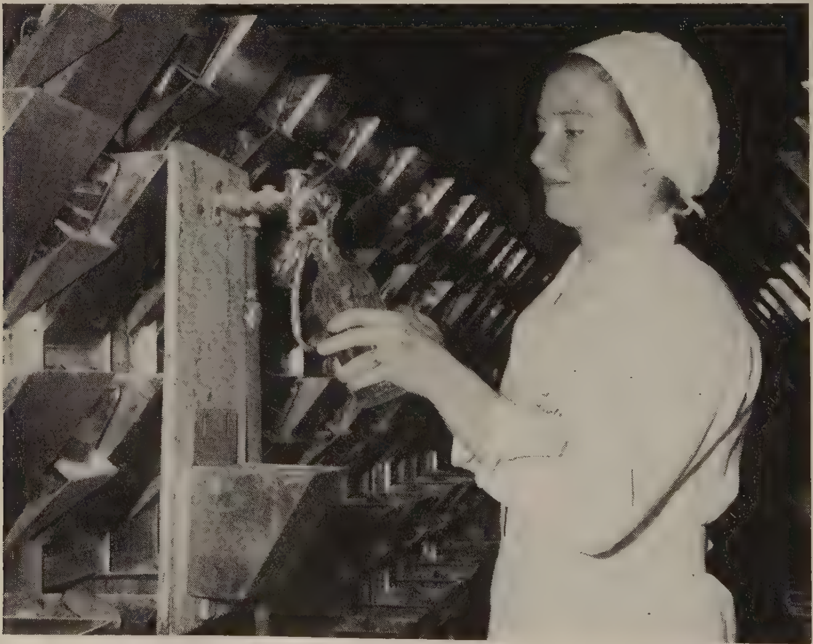

FIGURE 5. Animal technician wearing sterilized cap and frock. She is filling water bottles at the specially designed bottle filler. Experienced persons often perform this operation at the rate of from 180 to 200 bottles per hour.

Cages. All of our holding cages are of combination galvanized and stainless steel. The breeding and maternity cages are all of stainless steel. The enduring life of stainless steel, as well as its ease of cleaning, warrant investment in these permanent cages. Our cages were specially fabricated, and their design incorporated 10 years of experience in cage remodeling, as well as ideas obtained in the field. The holding stock cages are 14 × 14 × 9 inches in batteries 5 high, while the maternity cages are 10 × 15 × 9, also in batteries of 5. Units of 5 breeding cages are 24 × 24 × 9 inches, again in batteries of 5. The cages are standardized in design and differ only in dimension. The door opens in the manner of an overhead garage door, being contained within while the cage is open. Since the door rotates on stainless steel pins, no maintenance is required. The floors of the cages are permanent, watertight stainless steel pans. Since we operate under a barrier system with the animals free of infection, there is no need for removal of the pans. Cleaning and animal selection are accomplished from the front of the cage, while feeding and watering can be done simultaneously from the rear service aisle. Blackboard paint on the fronts of the cages permits identification of animals within each cage to be made with chalk, and subsequently erased with a sponge when new animals are housed.

Summary

Many basic concepts have been established in the production of disease-free vertebrates. However, with the absence of controlled experimental data, they cannot positively be stated as correct or as the only method to accomplish this aim.

A barrier system has been generally accepted for the operation of production of large colonies. Everything within this barrier has been previously sterilized and, once set in motion, all materials subsequently entering are rendered free of pathogens and parasites.

A basic construction of masonry and steel with nonporous interior surfaces, is a primary prerequisite. The provision of a minimum number of doors and no windows constitutes added insurance for exclusion of wild rodents and vermin. Air conditioning with provisions for bacterial filtration or destruction, proper levels of humidification, and maximum air change have been generally accepted.

Dry heat, steam, and germicidal gas are used currently to sterilize bedding materials. Due to the minimum impairment of nutrients, pasteurization (600° F. for 7 min. in a dry oven) has proved to be the method of choice to insure destruction of pathogens and parasitic forms.

Personnel must pass through a series of locks for removal of outer clothing and scrubbing or showering; then they don sterilized garments. Even with all reasonable precautions taken, the caretaker is still felt to be the greatest single hazard. More than one breeding unit with one or two caretakers seems preferable.

Selective breeding or testing and eradication techniques within an infected population seem to reduce infections and parasitism rather than eliminate

them. Derivation of initial breeding stock by cesarean section and hand feeding or foster suckling on known clean dams has been proved to be the only practicable starting point for a disease-free colony.

It has been our experience, as well as that of others engaged in the production of disease-free animals, that minimum losses and increased fertility, coupled with a more uniform and rapid growth, reduce the direct costs per animal. Once the initial capital investment for the physical facilities has been amortized, it is felt that disease-free animals will cost the same as, or very little more than, conventional animals.

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Part II. Rearing Germfree Vertebrates

DETERMINATION OF GERMFREE STATUS*

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Introduction

The determination of the microbiological status of animals maintained by ermfree techniques presents a complex and challenging problem to the microbiologist. Germfree technology, by the implications of the term, must separate the animal from a variety of microbial forms found in close association with the host during its existence in nature. Bacteria, yeasts, molds, actinomycetes, rickettsia, viruses, protozoa, worms, and arthropods are all known to stablish varying degrees of association with the host, ranging from obligate parasitism to only seemingly casual association. The ability of cesarotomy to solate the host completely from intimately associated forms of life presents an mportant problem.

The determination of germfree status requires microscopic or culture methods or both for the detection of the previously mentioned forms. The complexity of the test procedures varies with the organism sought. Routine bacteriological and mycological culture methods employing artificial culture media are used to detect the presence of the more common and more readily ultivated bacterial and mycological forms. The addition of body fluids or ther supplements to artificial media aids in the cultivation of more fastidious rganisms that require enrichment for growth. The detection of organisms hat are difficult to cultivate or whose growth is obligately dependent on other living cells calls for still more complex methods involving animal inoculation r tissue culture. Finally, there are those organisms that have resisted all fforts at cultivation to date. It is necessary to depend solely on microscopic bservation to detect their presence provided, of course, that they are visible nder the microscope and are present in sufficient numbers to detect and recognize. Organisms that do not meet any of these requirements and produce no etectable effect upon the host must be considered inapparent contaminants. Animals that harbor only such "silent" contaminants will pass as germfree ntil such time as: (1) new techniques of detection are developed or (2) a shift in the equilibrium of the host-contaminant relationship in favor of the microrganism allows detection by some recognizable means.

FIGURE 1 presents a general scheme for determining the germfree status of n animal. The culture methods shown in the upper two quadrants of the cheme generally meet the nutritional, oxygen, and temperature requirements for growth of most bacterial and mycologic forms that have the greatest probaility of gaining entry to the germfree apparatus through a fault in the mechan-

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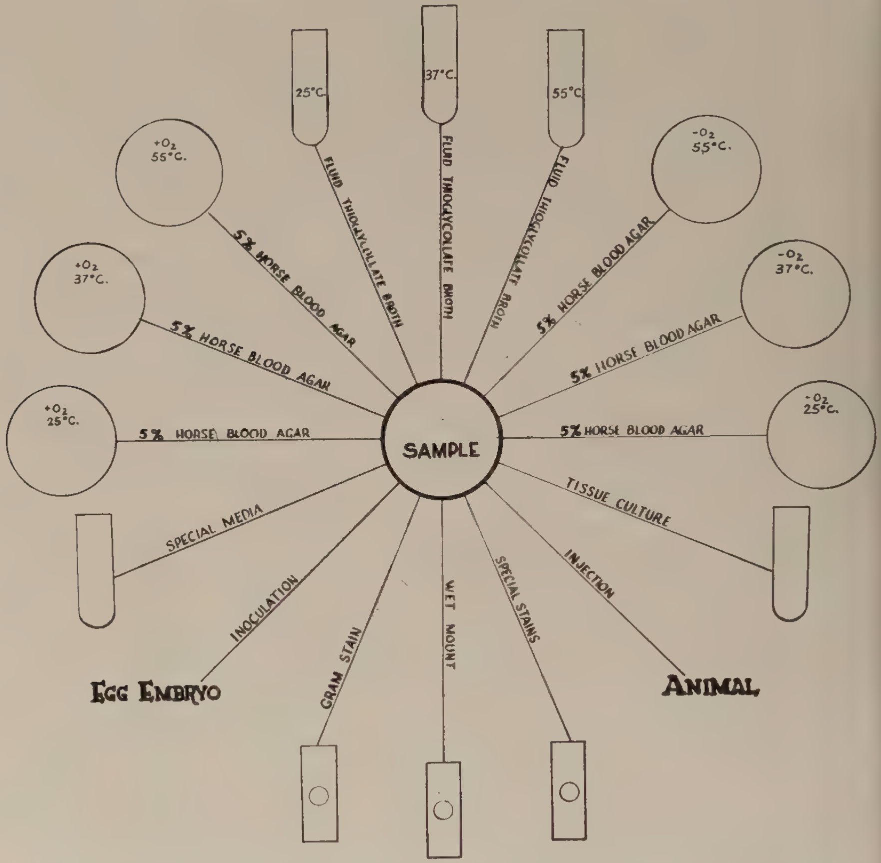

FIGURE 1. General scheme for the microbiological determination of the germfree status of animals. Key to samples: (1) feces, (2) urine, (3) skin, fur, or feathers, (4) body orifices, (5) food and water, (6) interior surfaces of cage, (7) interior surfaces of gloves, (8) organs and tissues, and (9) blood.

ical barriers. However, experience also has shown that the animal itself, particularly its alimentary tract, as well as the accumulation of voided feces and urine, and spilled food and water at the bottom of an isolation unit afford an excellent medium and a variety of environmental conditions for the growth of microorganisms. Direct microscopic examination of wet mounts or stained smears from these samples often may reveal the presence of contaminants if they are present in fairly large numbers. However, when only few microorganisms are seen, the question arises as to whether these forms are living or dead. Observation of motility in the wet mounts may quickly settle this question, but the presence of only nonmotile forms requires further proof. It is known that the diets fed to germfree animals contain microorganisms prior to sterilization. The sterilized diet may thus contribute small numbers of dead but nevertheless morphologically intact microorganisms that are visible in

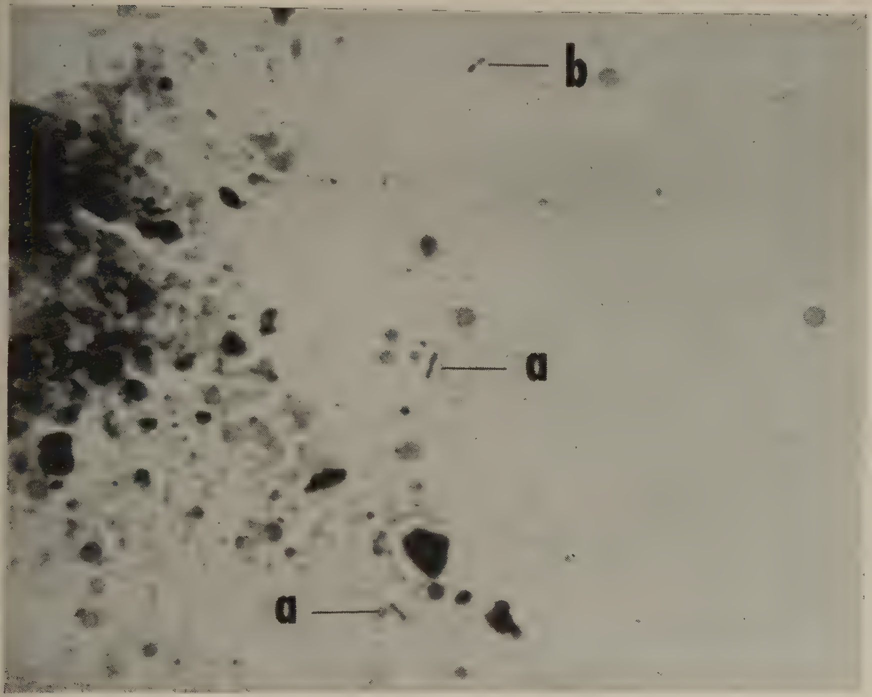

FIGURE 2. Rare incidence of nonliving bacterialike forms in the feces of germfree rats: (*a*) rodlike forms; (*b*) *Diplococcus*. The remainder is fecal debris, globules, and so on. Culture negative. Gram stain. ×950.

rare incidence upon microscopic examination of the intestinal contents (FIGURE 2). These animals yield negative cultures. Animals fed an abundance of nonliving microorganisms (such as autoclaved yeast) incorporated in the diet show an abundance of these undigested cells in the feces (FIGURE 3). Cultures of these feces are also negative. Microscopically, it is not possible to distinguish the bacteria in FIGURE 2 from those in FIGURE 4, which are from the feces of an animal monocontaminated with a viable organism that grew only poorly in the animal's alimentary tract. The eventual differentiation between the two examples depends on our ability to grow the organisms in culture. FIGURE 5 shows an abundant incidence of cocci in feces of a rat harboring a viable pure culture of *Streptococcus fecalis*, as compared with the abundant mixed flora in feces from a conventional rat shown in FIGURE 6.

While the routine culture procedures employed are believed to be adequate for the cultivation of the more common microbial forms, specialized procedures directed toward the isolation and detection of the more esoteric forms are also necessary; these are discussed below under the individual groups of organisms.

There are two inherent limitations on attempts to establish the germfree status of an animal. First, the term germfree, like the term sterility, can be

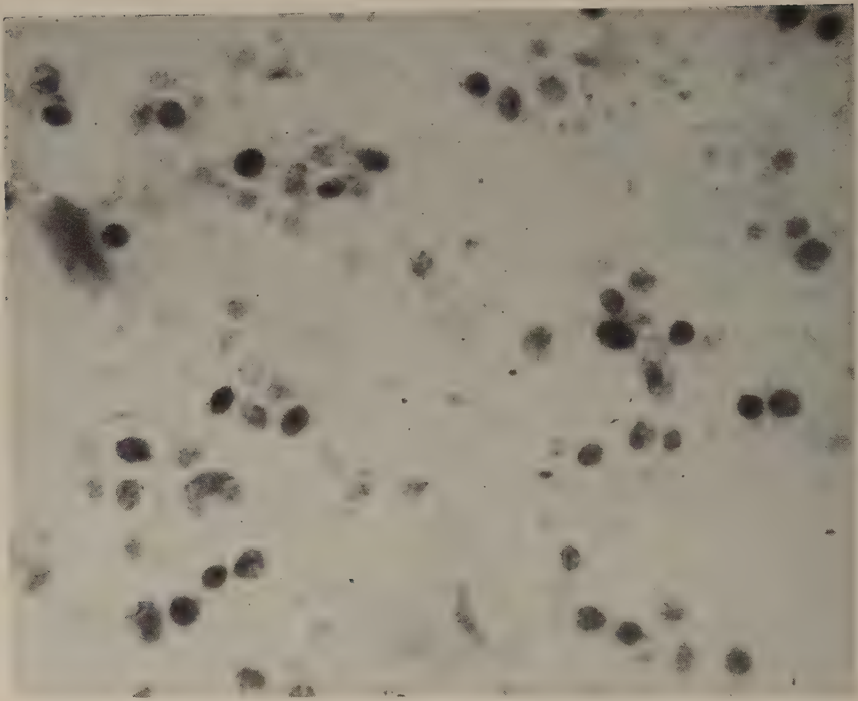

FIGURE 3. Abundant incidence of nonliving yeast cells in the feces of germfree guinea pig fed autoclaved yeast in the diet. Cultures negative. Gram stain. ×950.

measured only in terms of failure to demonstrate viable forms exclusive of the animal under test. The term germfree can be applied only when accepted procedures have failed to produce evidence of contamination. However, new organisms and new methods for their detection are continually being discovered. Thus, at any given period the germfree state can be regarded only as a relative state based upon the available and applicable test methods. Second, for practical reasons, it is obvious that every possible test cannot be applied to each individual animal used in an experiment. It would also be an exception rather than the rule for any single laboratory to have personnel available who have specialized in the detection and recognition of all the various groups of organisms that may contaminate an animal.

On this basis Trexler and Reynolds[1] have proposed the concept of an alpha-type operation for rearing colonies of germfree animals. Essentially, alpha operation involves the rearing of germfree animal colonies through generations in a sterile environment. The integrity of the isolation barriers that contain the sterile environment must be complete against structural or operational defects as measured by physical means as well as by biological tests on the animals themselves. Colonies that depend only on biological tests for the measurement of the security of operation would weed out animals contaminated

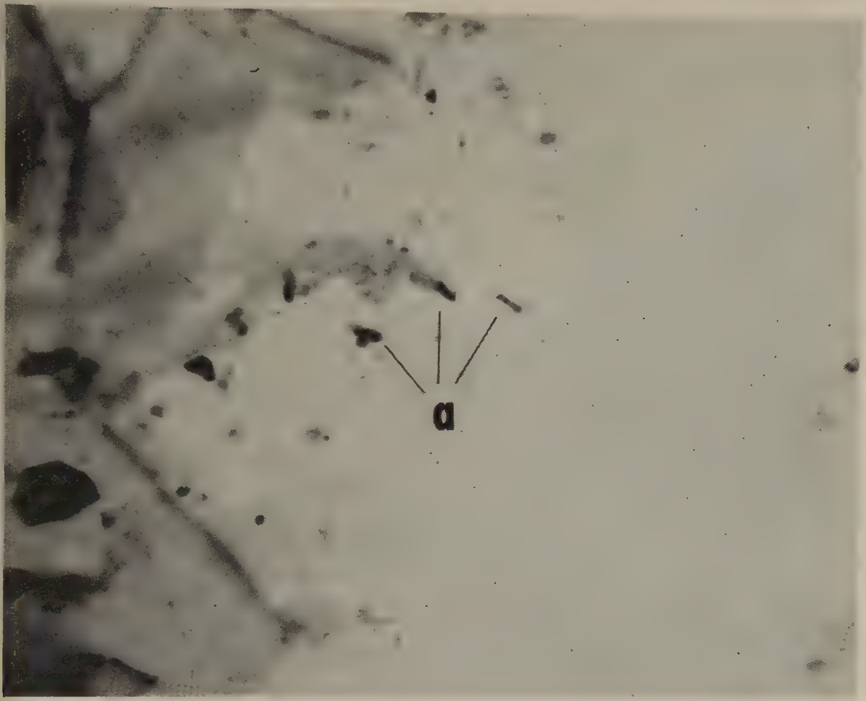

FIGURE 4. Rare incidence of viable bacteria (*a*) growing only poorly as a monocontaminant in the intestinal tract of "germfree" rats. Cultures positive. Gram stain. ×950.

with readily detectable organisms, but might at the same time select and accumulate animals harboring inapparent contaminants. These contaminants might become overt only later as they adapt to their environment. Under alpha-type operation a history of examinations for various types of suspected contaminants would accumulate to form a "pedigree" for the strain. This pedigree would remain intact for users of the animal strain as long as the integrity of the barriers was sustained and microbiological tests remained negative. Under these conditions it would not be necessary to run exhaustive tests on each individual animal in an experiment, but only on representative numbers of the colony. Trexler and Reynolds[1] have mentioned the operation of isolators of plastic film in a sterile room. A sterile room for maintaining alpha-type operation of germfree animal colonies is currently being constructed.[2]

The remainder of this paper is devoted to a discussion of the methods used for the determination of the germfree status of animal colonies and some of the experiences that have been encountered during their application.

Detection of Bacteria, Yeasts, Molds, and Actinomycetes

The routine microscopic and culture methods presented in FIGURE 1 are generally adequate for detection of organisms that can be readily cultivated.

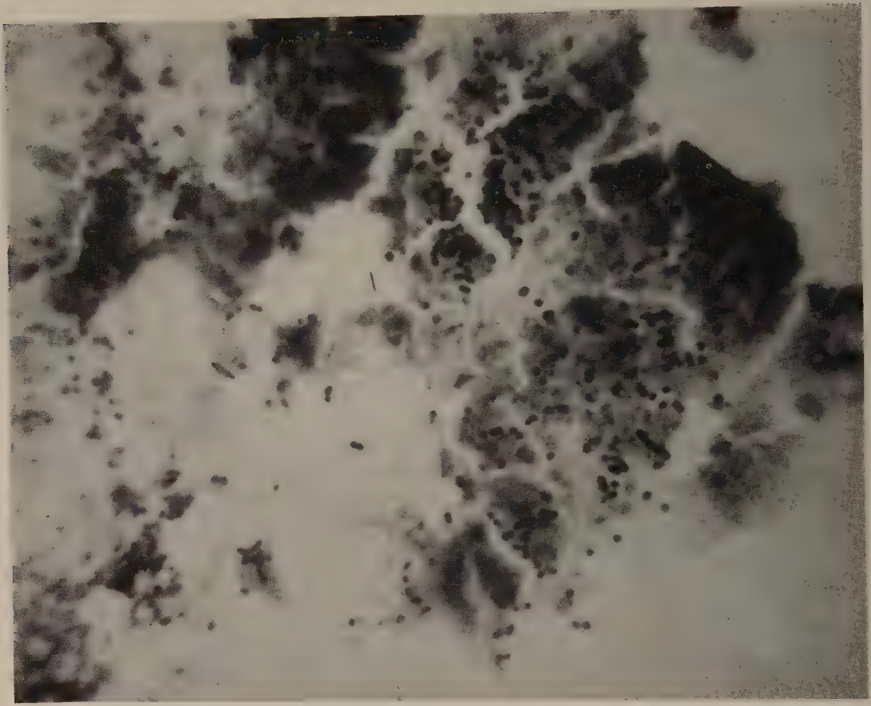

FIGURE 5. Numerous cocci growing abundantly in the feces of "germfree" rats harboring a viable pure-culture contamination of *Streptococcus fecalis*. Gram stain. ×950.

Spot checks for special groups of more fastidious bacteria such as pleuropnuemonialike organisms,[3] mycobacteria,[4, 5] and bacteroides[6] are also made, using appropriate media and environmental conditions.

Of particular importance is the occasional isolation of organisms that grow but poorly *in vivo* or *in vitro* or that can be isolated only from certain areas within the animal or isolation unit. By way of example, experience with a slow-growing Gram-positive pleomorphic anaerobic organism is cited. This organism eventually was detected in the entire germfree rat colony housed in Reyniers germfree (RGF) units[7] at the Lobund Institute in 1953. It became necessary to discontinue the colony and start a new line. Suspicion of contamination was first aroused when the numbers of bacteria seen in fecal smears seemed to increase. However, the incidence of cells in the smears never approached the numbers seen in frank contamination with the more common types of bacteria, and the inoculation of feces into routine culture media, into Maitland-type tissue cultures, and into embryonated chicken eggs failed to give positive cultures. Later, occasional sparse growth was observed in fluid thioglycollate medium inoculated with fresh feces, but replicate inoculated tubes did not always show growth, nor could growth be consistently confirmed by follow-up cultures. The sporadic positive cultures were thought at first

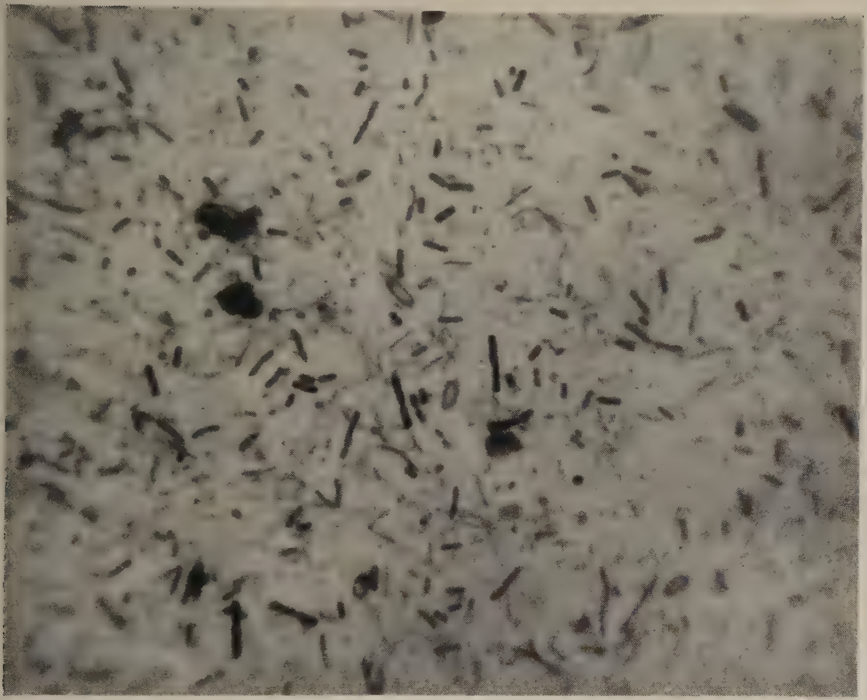

FIGURE 6. An abundant mixed flora in feces from conventional rats. Gram-positive cells show up dark; Gram-negative cells light. Gram stain. ×950.

to be accidental technical contaminations introduced during the inoculation of the medium in the laboratory. However, accumulating reports on observations of this type revealed highly pleomorphic Gram-positive bacteria recovered in each case. The sporadic but recurrent recovery of this same organism made it impossible to ascribe it to coincidence in accidental laboratory contamination. Furthermore, media taken into the RGF units and inoculated with fresh feces *in situ* also showed sporadic positive cultures of the same pleomorphic form. Finally, 25 rats from 13 different RGF units housing the rat colony were sacrificed and various organs or their contents cultured in fluid thioglycollate medium. Freshly voided feces, as well as accumulated waste in the RGF unit, were also similarly cultured. The results are presented in TABLE 1.

In laboratory parlance, the pleomorphic contaminant was referred to as the "cecal" organism (coded 56D10) because of the fact that we could obtain 100 per cent positive cultures from the cecum only.

It is interesting to note the sequence of events that led to the eventual proof of contamination. The earliest observations were characterized by the increased incidence of bacteria in direct microscopic examination of feces, but at first positive cultures could not be obtained from the same material. This

TABLE 1

ISOLATION INCIDENCE OF A GRAM-POSITIVE PLEOMORPHIC BACTERIAL CONTAMINANT (56D10) FROM A SUPPOSEDLY GERMFREE RAT COLONY

No. of cages	No. of rats cultured	Samples cultured*			
		Freshly voided feces		Cecal contents	
		positive/total	% positive	positive/total	% positive
13	25	9/24	37.5	25/25	100

* The organism was never recovered from blood, lung, liver, spleen, kidney, stomach, or small intestine, nor from accumulated feces, urine, and waste food at bottom of the RGF units. Cultures from the mouth, body surfaces, and cage surfaces were also uniformly negative.

was followed by a period of time during which sporadic positive cultures were obtained. The growth appeared as a light turbidity in fluid thioglycollate medium, but only after 7 to 10 days of incubation at 37° C. In later observations, the incubation time of the primary cultures decreased to 2 days, and the frequency of positive isolations appeared to increase. At no time was primary isolation made on aerobic and anaerobic streak plates, using blood agar or other solid media. However, growth on solid media could be accomplished by subculturing positive primary fluid thioglycollate cultures on anaerobic streak plates containing BBL Anaerobic Agar* or Bacto-Brain Heart Infusion Agar.† At the time the cecal cultures were made (TABLE 1), 37.5 per cent of the fresh fecal samples yielded positive recovery of the cecal organism. There appeared to be an adaptation of the organism to the animal host as well as a simultaneous adaptation in its ability to grow in laboratory media, although the latter may have been only a function of the size of the inoculum.

Finally, one may speculate on the original source of this contaminant, since it appeared in each of 13 RGF units housing the "germfree" rat colony. Indications are that the contaminant may have been introduced early in the history of the colony. One possibility is that the contaminant was originally introduced with the fetuses that formed the first generation, or that an accidental contamination took place at some time in the early history of the animals when the small rat colony nucleus was maintained in a single RGF unit. The progeny of this first and its succeeding generations were distributed to 13 different RGF units as the colony expanded. Another possible explanation for the spread of the contaminant may be the interchange of animals made among RGF units in order to improve the breeding performance of the colony. These transfers were carried out approximately 6 months prior to the time contamination was recognized. Regardless of which explanation might apply, it is quite evident that the cecal organism was carried as a "silent" or inapparent contamination in the colony for a considerable length of time. It would seem highly improbable that 13 independent accidental contaminations would result in the same fastidious contaminant in each case.

* Product of Baltimore Biological Laboratory, Inc., Baltimore, Md.
† Product of Difco Laboratories, Detroit, Mich.

The above description has been used to illustrate what was apparently a latent bacterial contamination that later became overt. Time played an important role in the eventual detection of the contaminant. The example may also serve to illustrate the need for examining more than voided feces and accumulated waste in the unit in order to determine the bacteriological status of the animals. Finally, the danger of interchange of animals between isolation units housing germfree colonies is illustrated.

Protozoa. Examinations for intestinal protozoa are made routinely in wet mounts prepared from voided feces. When animals are sacrificed, examination of intestinal contents, particularly of the cecum, are made since, in conventional animals at least, amoebae, flagellates, and ciliates are more readily seen in intestinal contents than in voided feces. Occasionally smears fixed in Schaudinn's fixative[8] are stained with Heidenhain's iron-alum hematoxylin[8] and examined for the presence of protozoa. Concentration methods by centrifugation and zinc sulfate flotation[9] are also used occasionally for the detection of small numbers of protozoan cysts that ordinarily might be missed in the routine diluted wet-mount examinations. Blood smears are stained with Wright's stain[8] and examined for blood parasites.

Protozoa apparently do not play an important role as contaminants in germfree animals. In our experience, thousands of fecal wet mounts and smears have been examined from rats, mice, guinea pigs, rabbits, and chickens without any evidence of contamination with protozoa, despite the fact that the conventional progenitors of the germfree stock may harbor an abundance of these forms.

Protozoa, as a group, do not appear to be likely contaminants per se once it is reasonably certain that none have been carried over during the cesarean operation. Any postoperative break in the isolation barriers would lead more probably to bacterial, yeast, or mold contamination, since these forms generally would be present in much greater numbers than protozoa in the environment outside the isolators. The most important tests for protozoa seem to be in the first generation, where carry-over from the mother during the cesarean section would be the most likely source of contamination. In no case have protozoa been observed.

Worms. Examination for intestinal worms is done macroscopically and under magnification. Wet mounts are examined for presence of ova. Zinc sulfate flotation methods[9] described for the concentration of protozoan cysts are also used for detection of ova and small larvae. At the Lobund Institute worm infestations have been found in no germfree animals except dogs. Phillips[10] found *Toxocara canis* and *Ancyclostoma caninum* larvae in puppies taken by cesarean section and sacrificed at birth in the RGF unit. Other puppies were sacrificed at approximately 2 and 4 months. One experiment ran for 28 days free from microbial contaminants, but was later accidentally contaminated with bacteria. Mature *Toxocara canis* were recovered from the intestines of these animals at sacrifice. Eggs were not detected in the feces of the puppies during the course of the run, nor were eggs seen in the vulva of a female worm recovered at autopsy. It is not known whether the absence of egg production was attributable to the environment during the maturation of the worm or whether the parasite itself was atypical and incapable of producing eggs. It must be assumed that the young dogs were infected from the conventional mother *in utero* before the cesarean section was performed. The bacterial contaminants that were detected later in the experiment gained access to the RGF unit through an accidental break in the isolation barrier, but this did not occur until several weeks following the cesarean section. It is interesting to note that dogs could be obtained that initially harbored worms but no microbial contaminants. The inability to obtain germfree dogs free from worms will probably necessiate the use of gravid females that have been dewormed and maintained free of reinfestation prior to and during pregnancy before cesarotomy is performed.

Arthropods. Animals are examined grossly for evidence of infestations with lice, fleas, or mites. None has ever been found. It seems highly unlikely that arthropod parasites would gain entry to germfree animal colonies either during the cesarean section or through accidental breaks in the isolation barriers.

Rickettsiae. The search for rickettsiae in our germfree colonies has not been conducted on a routine basis. Occasional spot checks have been made on intestinal contents, and impression smears from the spleen and other organs have been stained differentially with Macchiavello's[11] stain and examined for rickettsiae. The presence of these forms has never been established in any of the examinations made.

Viruses. Until fairly recently, demonstration of animal virus has depended mainly on the overt manifestation of clinical disease or pathology in a host animal or embryo. On this basis, germfree animals that showed no outward manifestations of disease and that delivered normal young through successive generations were assumed to be virus free. Questionable as this assumption might be, it was supported by preliminary observations that germfree animals might reveal virus infections more readily than conventional animals. If this were true, then germfree animals might show overt infection with viruses that remain masked or silent in conventional stock. One example of presumptive evidence may be taken from Gordon *et al.*[12] A spontaneous disease occurred in germfree chickens, but not in birds hatched under conventional conditions from the same clutch of eggs. The syndrome involved the central nervous system of germfree chicks only and consisted clinically of severe body tremors, weakness, and high mortality. Histologically, the disease was marked by a proliferation of the meninges in the region of the cerebellum and medulla that caused a derangement of the adjacent nervous tissue. While, for technical reasons, a viral agent was not demonstrated in these animals, evidence was collected to show that the etiology of the syndrome was probably not due to nutritional, chemical, or environmental factors peculiar to the germfree isolation. These animals tested free of bacteria and fungi. The consensus of several experts who examined the histological preparations was that the possibility and, even the probability of a viral agent could not be excluded. Other unpublished evidence indicates that respiratory disease of presumably viral nature may at times have been carried in rats and guinea pigs that were taken by cesarean section and that tested free of bacteria and fungi. Nevertheless, by selection of only normal asymptomatic stock, it seemed possible to obtain virus-free animals.

The recent strides that have been made in tissue culture techniques and the application to the growth, primary isolation, and identification of viruses has opened new methods for virus detection. Of particular importance is the discovery of viruses that remain asymptomatic in the host, but may manifest themselves by their cytopathogenic effects in tissue culture. A line of approach to detecting virus in germfree animals has been to inoculate organ homogenates from germfree animals into established lines of tissue culture, egg embryos, and suckling mice. Ward, in a personal communication, has reported preliminary findings that ECHO or orphan-type viruses cytopathogenic for monkey kidney cells have been isolated from the cecum of several animals in a group of 17 germfree rats examined. However, the results were not unequivocal and bear repeating under more stringent conditions of sample isolation. A basic disadvantage is recognized in the standard tissue culture line of approach. Lennette,[14] Melnick,[15] and others have pointed out that even carefully cloned tissue culture cell lines and the serum employed in their propogation may contain their own viruses carried over from the donor. It is therefore necessary to prove whether a virus, particularly an unknown one demonstrated in this manner, has originated from the inoculum under test or from the test system itself. Attempts[13, 16] are being made presently to establish cell lines from germfree animal tissues. Successful propagation of tissue cultures of this type, particularly in the presence of germfree, rather than conventional animal serum, would circumvent the previously mentioned pitfalls, and the demonstration of indigenous agents in these cultures by cytopathogenic effects or other means would be more specifically convincing.

Finally, the application of stress or forcing techniques has been used in attempts to make germfree animals reveal their silent contaminants, if present. Ward,[13] in preliminary findings, has been able to produce lung consolidation in germfree rats by the serial passage of lung tissue. However, an agent has not been isolated and identified to date. Reyniers,[17] working with other isolated segments of the germfree rat colony and with germfree mice, has failed to produce lung consolidation by similar forcing techniques.

Reyniers and Sacksteder, elsewhere in this monograph, propose the use of carcinogens as a method of stress to bring out suspected agents of disease that do not occur spontaneously; for example, carcinogen induced tumors can be examined for transmissible subcellular particulates. Kassel and Rottino,[18] in discussing the question of viral etiology in Hodgkin's disease, have presented the problem of particulates in a way that can be applied generally to this discussion: "The problem faced by others consists in proving the particulate matter, demonstrated by physical methods to be living. Or, in the event a living agent—a virus—is isolated, to prove that it was obtained from the patient and not from the complex living systems employed to isolate it. . . . However, the search must go on, for even 'negative' results are affirmative in that they show up the investigational approaches which will prove unprofitable."[18] This last sentence may be reversed for our purposes. If some of the animals we have called germfree are indeed not virus-free, then selection of the negative reactors may be a profitable approach to obtain virus-free stock. The problem of whether an agent comes from the patient or the biological test system is eliminated, since both have been locked in the confines of the isolation environment and may, in some cases, originate from the same individual. In the final analysis, the best criteria for determination of the germfree status, particularly in the search for inapparent, masked, silent, or latent contaminants, or whatever other name one may apply to these agents, will come from the use of the germfree animal itself as the test system or from test systems derived from germfree stock.

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Discussion of the Paper

Question: Have tests been made for parasites, particularly nematodes? If so, what is the route of infection? Particularly, how can we account for the presence of nematodes in the fetus?

WAGNER: Tests for parasites such as nematodes are done by searching for ova in wet mounts made from the feces. The search for ova is done routinely at each culture period. Occasionally, concentration methods by flotation in $ZnSO_4$ are used to supplement the direct wet-mount examination. As I mentioned in my paper, we have found no worm infestations in our germfree animals, with the exception of dogs. Phillips has found *Toxocara canis* in puppies delivered by cesarean section into a germfree environment. However, the puppies that harbored these worms never voided ova in the feces, nor were mature ova found in the female worms recovered when the puppies were autopsied.

The infection was undoubtedly prenatal. Larvae of *Toxocara*, like those of *Ascaris*, have a tissue phase in which they penetrate the intestinal wall and are usually carried by the circulation to the liver and finally escape via the lung. However, other routes, such as the urogenital tract, are also known to be subject to such penetration. This would account for the presence of nematodes in the fetus by the prenatal infection of the mother.

The worms found in the puppies were not tested for contamination because they were not detected until the animals were removed from the germfree isolator and autopsied; in other words, the post-mortem was conducted outside of the germfree system. During the time the animals were alive inside the isolator there was no evidence that worms were present, since no ova were found in the stools. It is our assumption that the worms were present without bacterial contamination during the early stages of the experiment. Later, the animals became accidentally contaminated with bacteria while still in the confines of the isolator. It is assumed that the worms also became contaminated at this time.

I am sure that we have not covered all of the microbiota that are known to exist. Certainly, many forms have not yet been cultivated on artificial media. We depend principally upon the microscopic examination of tissues, feces, and so on to detect forms that are difficult to culture.

THEORETICAL AND PRACTICAL CONSIDERATIONS OF GERMFREE ANIMALS IN VIROLOGY

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Recent reviews[1-7] and current experimental work designed to study the relationship of neoplasms or cancer to viruses have stressed the need for a broader and more critical approach to the problem. Stanley[8] has pleaded for the acceptance of the working idea that viruses are the etiological agents of neoplasms, including human cancer. Many have questioned such an approach, but the fact remains that under appropriate conditions tumors and other neoplastic growths in animals have been shown to be produced by viruslike agents.

The widely diversified efforts that are being made to find conclusive evidence of a causal relation between virus and cancer are hampered by the great complexity of factors that must be considered. Bryan[3, 9] has stressed the wide variation in the susceptibility of different types of hosts to their respective specific tumor viruses. This relation is characterized by an interplay between the quantity of virus inoculated and the level of host susceptibility in determining response. The host susceptibility is influenced by the age of the animal, by hormonal balance, by genetic background, and by many other factors. Duran-Reynals[10] has stressed his belief that not only is the age of the host important, but also the age of the donor animals. With the mouse tumor agent of Bittner,[11] all of these factors have been shown to influence response rate.

In order to say that a viral agent is an etiological factor, it has long been considered that such an agent must be "demonstrable"; that is, we must isolate it from the diseased tissue or animal and, following purification, be able to characterize it chemically, physically, and biologically as a distinct entity. Furthermore, we must be able to relate the isolated agent specifically to the original disease. Beard[12] has spelled out the complexities of the problems of purification and isolation and the necessity for a systematic application of methods of analysis, both physical and biological. He has pointed out especially that isolation of a viral agent from intact tissue sources is a wasteful process; only a small proportion of the actual quantity present can be recovered for study. In general, only the larger agents, or those where the relative mass of virus present is large, have been successfully isolated and analyzed. Beard also stressed that isolation of agents and the attendant fruitful analyses are likely to be attained by a persistent trial-and-error approach with a combination of procedures; the choice and sequence of combinations utilized are dictated to a marked degree by the character of the materials available for study. The rigorous and well-known disciplines established for identification, characterization, and analysis should not, and cannot, be supplanted by electron microscopy. This discipline is only one of the many recently developed that have given us the opportunity to push our studies forward more rapidly. The utilization of ultrathin sections of cells diseased with viruses affords the opportunity for the identification of intracellular virus, but unequivocal interpretation of such findings depends upon the results of the classic identification procedures outside the cell.

The work with the leukemias, reviewed recently,[13] has shown that the problem of masking is one of low host sensitivity or reactivity rather than of hidden virus. The host factors control the invasion and provide the materials and processes required for the multiplication of an agent and its manifest response. Even with the tremendous impact that the continuously expanding tissue culture approach has made in recent years and its application to the analyses of various host-virus systems, the emphasis is still predominantly on virus activity rather than on the more inclusive concept that the events that occur are the result of the interaction between the agent and the host cell. In this respect it should be evident that the host cell is the determinant; in reality, the virus must be considered to be a relatively fixed entity entirely dependent upon the host, first for acceptance and growth and, finally, for manifestation of its presence. This manifestation may be grossly apparent in the form of a neoplasm or a disease, or it may remain dormant and be detectable only by special means.[14]

Although it has been established that many viruses can coexist in harmony with the host in latent form through many generations,[11, 15] there may be some basic nonpathogenic cellular entity which, under proper chemical or physical stress or stimulus[8] is activated to become a viruslike or neoplastic agent. Whether the disease entity is considered a mutable gene or a cell particulate does not really matter. It is becoming more and more apparent that the virus-host problem as a whole is essentially a cellular one; even the poliovirus, which is only 30 mμ in diameter, exhibits distinct cellular characteristics.[16, 17] This virus, like other animal viruses, is comprised of an outer membranous structure surrounding a plasm that contains an internal nucleuslike body. The biological activities (infectiousness and antigenicity) undoubtedly require the intact particle for a full expression of their capacities. However, it has been shown repeatedly that the capacity of animal viruses to incite infection and virus multiplication resides in the nucleic acid portion, whereas the specific antigenic properties seem to be associated with the protein moiety and are determined by it. Therefore the host-virus relationship, where so much definitive information is needed, can be considered basically to be one of cell versus cell; in some cases there is harmony, in others cold war and, in still others, complete annihilation of the host. In the future progressive analyses of these intricate associations between infectious agents and their hosts it seems likely that certain concepts and even whole philosophies must change.

The germfree animal[18] would seemingly provide a test host which, if properly used in this broader and more critical experimental approach that we are considering, could provide useful information relative to virus-host relationships. The germfree animal is known to be free from life other than that produced by its own protoplasm, and it exists in a sterile environment. This would produce a stable base line for experimental studies with respect to the absence of the usual spectrum of antibodies to microbial flora. In this category young germfree animals could be compared to older ones and to conventional animals, to obtain relative sensitivity data. The suckling mouse has proved to be a valuable adjunct in the study of such viruses as the Coxsackie and ECHO (enteric) groups.[19] This work suggests that the germfree animal, protected as it is from infective microorganisms, might provide a more sensitive test host. It may be reasoned that the sensitivity of the suckling mouse to certain agents could be attributed to its relative inexperience with pathogens and the resulting complex antigen-antibody situation due to such experience. In brief, the germfree animal can be pictured as a relatively stable basic host where the response to a given agent or stimulus can be observed uncomplicated by the response to or interference from other natural pathogens.

In the newly developing areas where tissue culture systems are employed in direct relation to animal studies, a wide variety of approaches utilizing germfree animals appears possible. A "primordial" tissue culture system free of unknown viral and intrinsic factors would be extremely useful. On the other hand, wide experience has shown that many of the so-called normal tissue culture cell lines that have been established from conventional animals, even from embryonic tissues, are, after a few passages, almost indistinguishable from those derived from highly malignant cancers.[1] Most certainly there must be some significant differences between tissue culture cells derived from normal tissues and from malignant ones, at least initially. It is in such areas that studies directed toward the elucidation of the fundamental factors responsible for cell growth and metabolism and their relation to what appear to be the malignant transformations or their prevention could be pursued.

Our initial attempts to establish germfree chicken tissue cell lines, from both tumor and normal tissues, were not successful; the cells cease to grow after one or two transfers. Attempts to test the germfree Bantam chick tissues (spleen, liver, lung, pancreas, and muscle) for the presence of cytopathogenic agents were likewise unsuccessful. Monkey kidney, amnion Fl 80, HEp 2, established human heart, and Detroit-6 tissue culture cell lines were used, but no specific cytopathogenic effects have been observed through four passages.

Since sufficient quantities of germfree chicken sera have not been available for medium studies, additional experiments are now under way using established tissue culture lines maintained with horse, calf, or human sera treated with beta-propiolactone and ultraviolet irradiation.[20] It has been found that such treated sera are most satisfactory for the growth of Detroit-6 and HEp 2 cell lines. Peculiar and misleading effects such as nonspecific sloughing and cytotoxicity were often observed unless the sera used in the culture media were prescreened to eliminate such "toxic" sera. The beta-propiolactone treatment appears to eliminate the nonspecific "toxic" effects, while the ultraviolet irradiation is of sufficient intensity to eliminate known viral agents.

Much of our present information relative to a proposed viral etiology of neoplasia has been gained from studies dealing with the interaction of chemical carcinogens and Rous sarcoma, rabbit papilloma, and the fowl pox viruses. In broadening this spectrum, Reyniers for a number of years has been studying the effects of injecting small quantities of methylcholanthrene (MCA) into germfree chickens and producing tumors. A preliminary account of some of his experiences is presented elsewhere in this monograph. However, some of the background work that led to the collaborative efforts between the Parke-

Davis group and the investigators at the Lobund Institute, Notre Dame University, Notre Dame, Ind., during the past year will be described briefly.

The following may seem somewhat digressive, but it contains, I believe, documented material that is pertinent. In the early 1940s an extensive study was made of a normal chick embryo component that was found in preparations of equine encephalomyelitis (EE) virus grown in chick embryos.[21] This virus is about 50 mμ in diameter in contrast to a diameter of 15 to 25 mμ for the normal component. These two particles could not be separated centrifugally, but it was found that the latter could be almost completely eliminated from extracts of ground infectious embryo tissue suspended in balanced saline solutions at a *p*H of 7.5 to 8.0. The much more stable virus could then be sedimented and obtained in relatively pure form for chemical, physical, and biological characterization. The normal component[22] was found to be a ribonucleolipoprotein complex containing 10.5 per cent or more of ribonucleic acid, 35 per cent of total lipid; 40 to 41 per cent protein, and 7 per cent carbohydrate; 67 per cent of the total lipid was phospholipid. It was postulated at the time these studies were reported (1942) that the normal component appeared to have no specific relation to the EE virus, but that it was undoubtedly of physiological importance.

The work was later extended to include comparative studies of material from whole brain tissue of man, the rabbit, and the chick, both embryonic and adult.[23] Homogeneous preparations of spherical particles approximately 18 mμ in diameter were obtained from the embryonic tissue. These preparations gave sharp sedimentation boundaries with a sedimentation constant (S) of 70 to 75. The yield of the component from adult tissues was only one-fifth that obtained from the embryonic tissues, and the sedimentation diagrams were diffuse, indicating heterogeneity of particle size. The variations observed with age and the fact that preliminary fractionation studies of certain brain tumors of man indicated that they contained much higher quantities of a similarly sized particulate component than normal brain tissue, suggested that further study was indicated, especially with respect to the "investigation of noninfectious pathological processes such as neoplasms." This statement was made in 1943.[23]

At the Second National Biophysics Conference held in February 1958 in Cambridge, Mass., the structure and function of microsomal particles was discussed.[24] An attempt was made to define the nature and origin of such particles and to clarify the terminology used by various workers. The normal chick embryo component discussed above is in this same microsomal particle category.

In practice, the microsomal fraction refers to the pellet obtained after centrifuging cell extracts at 100,000 g for about 1 hour subsequent to a clearing centrifugation at 20,000 g for about one-half hour. The term microsomal fraction has been used to refer not only to this 100,000 g pellet, but also to various parts and subfractions of this pellet. It was decided to accept the definition of the microsomal fraction given above and to refer to a number of classes of particulate material that are present in this pellet as microsomal particles. It was the physical and chemical nature of these particles that attracted most attention.

Paul Zamecnik of Massachusetts General Hospital, Boston, Mass., Alfred Tissiéres of Harvard University, Boston, Mass., and George Palade of the Rockefeller Institute, New York, N. Y., discussed the nature of these particles at some length, and their reports may be summarized briefly as follows.

Within the last few years a number of workers have noted the existence of small, dense particles in living cells. These particles lie in the size range of 7.5 to 25.0 mμ in diameter; this, incidentally, constitutes the only criterion to date for classifying them. The particles have been referred to by several different names: Palade particles, ergastoplasmic granules, ribonucleic acid (RNA) particles, ribonucleoprotein (RNP) particles, and microsomal particles. Recent work indicates that these are all the same kind of particles visualized in different ways, either in the form of purified concentrates or in thin sections with the electron microscope. It appears that under proper conditions these particles can be isolated from all types of cells, although there is some variation in size and concentration from cell type to cell type with lesser variation within cells of a given type and some variation in size within individual cells.

Most of the ultrathin section work has been done with liver and pancreatic cells, but Palade warned against the unwarranted extension of these results to include all other cell types. However, similar particles can be found in *Escherichia coli* and yeast, to mention but two, and it is with material from these sources that most of the physical and chemical studies have been done.

Chemically, these particles are usually found to be about 50 per cent (by dry weight) RNA arranged as a central core surrounded by a protein coat that may or may not contain other material (for example, several workers have noted two distinct classes of particles in *E. coli*: one that contained RNA and protein, and another that contained, in addition, a lipoprotein). The method by which the particles are purified either chemically or physically appears to affect their structure and, to some extent, their chemical composition, presumably by removing more or less of the protein coat from the RNA core.

Functionally, these particles were clearly shown to be involved in protein synthesis in the cell, although it is not clear whether the RNA is formed before or at the same time as the protein. It was suggested that these particles carry the template or organizer for protein synthesis from smaller polypeptide units.

In addition, there were several other specific points of interest. Gillchriest[24] from the University of Wisconsin, Madison, Wis., isolated a particle from *Azotobacter vinelandii* cells that was stable in very low ionic strength buffers and had a sedimentation constant of 55 S. When the ionic strength is raised to 0.02 at *p*H 7.5, particles are dissociated to components of 30 to 40 S. This is of interest in view of the similarity of the results obtained in the early work with the "normal component" associated with EE virus.[22]

Dagley of Leeds, England reported on the concentration of a 40 S. particle from *E. coli*.[24] He studied this as a function of cellular growth, and the concentration was found to be increased during active cell division and slowly decreased after growth ceased. It was greatly reduced in cells incubated with incomplete growth media.

The thinking behind this work was not oriented toward the virus or cancer problem, and there were no reports of studies on material from tumor cells.

However, the work cited would lend presumptive evidence to the hypothesis that these particles are important, not only in protein synthesis and metabolism, but also in tumor induction and growth. In any case, there is no doubt that the "tumor-inducing factor" of Burton[25] and the "transmissible agent of leukemia" of Gross[5] are constituents of the microsomal fraction. The ultracentrifugal sequence described above for the separation of microsomal particles is approximately the same as that used in the EE virus-normal component work discussed earlier.

Such particulate materials in the size range of 10 to 25 mμ have literally dogged our footsteps throughout the studies associated with purification and characterization of many viruses, including the influenzas,[26, 27] Coxsackies,[28] foot-and-mouth disease,[29] polio[16, 30] and, particularly, the adenoviruses.[31, 32] It has been noted that adenovirus will grow especially well in tissue cultures of HeLa or HEp 2 cells, both derived from human carcinoma. In FIGURE 1, which shows adenovirus Type 3, it will be noted that the background of this electron micrograph is covered with uniform particles approximately 20 mμ in diameter; this is a characteristic feature of malignant tissue culture cell line extract concentrates. We see a great profusion of these 15- to 25-mμ particulates in practically all neoplastic cell extracts in comparison to extracts of normal cells. Monkey kidney cells contain such particulates, but in nowhere near the profusion observed in FIGURE 1; FIGURE 2 is an electron micrograph of a comparable concentrate from monkey kidney cells. This difference may be significant in the light of Dagley's report that the concentration of microsomal particles is increased during cell division.[24] In general, it may be said that these small particles are always prevalent in virus-infected and malignant cells; that is, wherever we encounter active cell division and metabolism.

Up to the present time, to the best of our knowledge, there has been no substantiated case where definite viruslike biological activity has been shown to be associated with particulates in this size range. As reported elsewhere in in this monograph by Taylor *et al.*, there is now at least presumptive evidence for such activity associated with tumorigenesis in germfree chickens. When normal germfree animal tissue is extracted and centrifugal concentrates prepared, only a very small proportion of 15 to 20 mμ particulate material is obtained. FIGURE 3 shows such an electron micrograph. When a carcinogen is introduced, there are large quantities of material in this size range produced in the tumors, and gross examination of extracts by means of electron microscopy indicates a progressive increase, with continued passage, in the quantity of this particulate material extractable per gram of tissue. FIGURE 4 is an electron micrograph of second-passage tumor microsomal particles. These particulates in tumor tissue, however, are stable in saline solutions; they do not disintegrate into unsedimentable material when extracted in saline, as did the normal chick embryo material studied earlier. These preliminary findings, it seems to me, make it imperative to carry out parallel studies in germfree animals and in appropriately integrated tissue culture systems in order to correlate and quantitate such studies. For example, if an agent responsible for the passage and growth of chemically induced tumors in germfree animals could

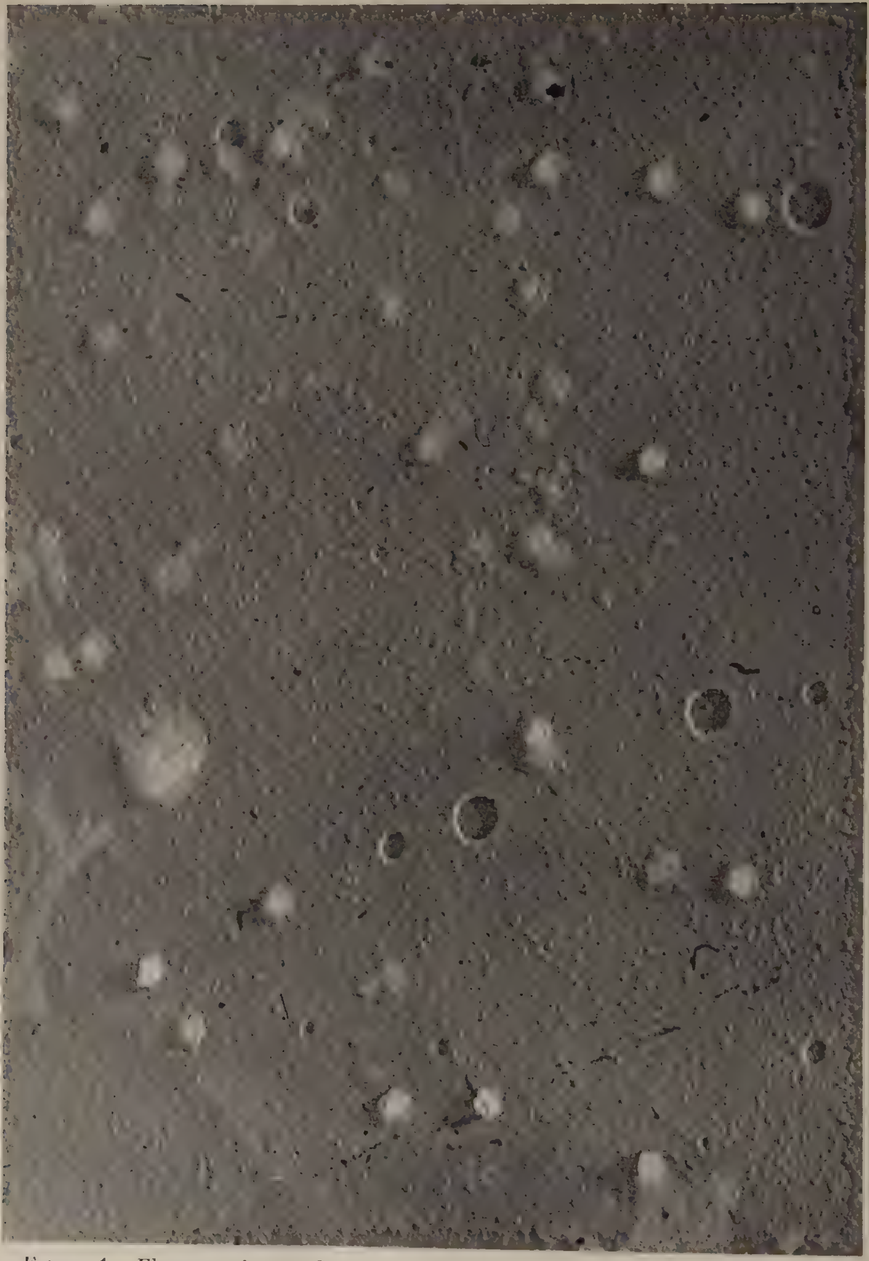

Figure 1. Electron micrograph of a concentrate of adenovirus grown in HEp 2 tissue culture. The large bodies are a form of the adenovirus particles. The profusion of 20-mμ particles associated with the virus is especially noteworthy and appears to be characteristic of extracts derived from malignant tissue culture cell lines. ×60,000.

FIGURE 2. Electron micrograph of a concentrate of an extract of uninfected monkey kidney tissue culture cells. The extracts used to prepare this micrograph and that of FIGURE 1 were comparable, but there is a notable absence of the 20-mμ particulates in the monkey kidney cell extract. ×60,000.

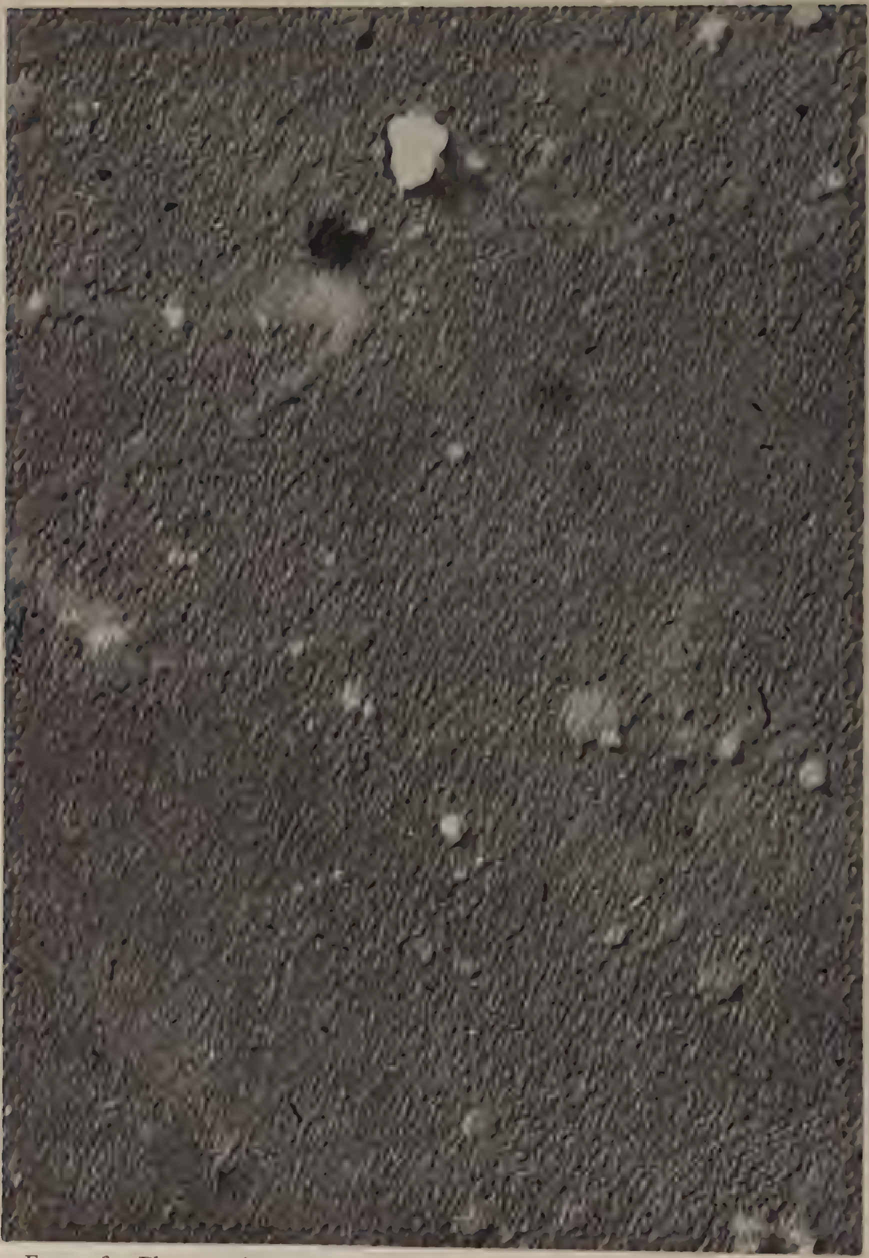

FIGURE 3. Electron micrograph of a concentrate of an extract of normal chicken breast muscle. As in FIGURE 2, there are very few microsomal (20 mμ) particles. ×60,000.

FIGURE 4. Electron micrograph of a concentrate of an extract of second-passage germfree chicken tumor microsomal particles. See Taylor *et al.* elsewhere in this monograph for details of preparation. ×60,000.

be adapted to grow in tissue culture, a more rapid method of analysis and characterization would be available.

Let us return to the consideration of the germfree animal as a host or medium for virus studies in general. Theoretically, the germfree animal, in contrast to the conventional animal, provides a more standardized host for the study of such infectious agents. The absence of microbial flora, including known or inactive viruses and their antibody patterns, may permit more uniform response to quantitated viral inocula. There are many areas in virology to which the germfree animal could be expected to contribute useful information: (1) the relationships of bacteriological and virus infections; (2) direct virus-to-virus relationships (for example, interference phenomena); and (3) carcinogens and tumors, which are discussed in some detail by Reyniers elsewhere in these pages. In conventional animals, in the area of the tumor agents as well as in other areas, many of the findings are somewhat confusing and difficult to analyze. Often animals must be primed with hormones or X ray in order to elicit a response, and even then such response is subject to wide variation. How much of this variation is caused by host resistance that may be due in part to experience with other agents or pathogens completely foreign to the agent under study? In general, the younger the host and the younger the donor animal from which the tumor (Rous sarcoma) is taken, the greater the quantity of virus that can be extracted. This could be an example in which the younger animal has had a limited pathological experience. When strong inocula are used, the maximum of extractable virus is produced in the resulting tumors. This could be an example where a sufficient quantity of agent inoculated overrides the natural resistance of the host. There may be natural inhibitors present or produced in the infectious process. Bryan[33] has given an excellent review of this subject in relation to the Rous sarcoma and other tumor agents. Inhibitory effects have been observed with other viruses such as influenza and Newcastle disease.

As more sensitive methods of detection have become available, there has been a steady increase in the recognition of new agents derived from apparently healthy hosts. Of especial interest at the present time are the viruses of the adenovirus group. These have been linked with a cytopathogenic effect on human cancer cells; further, certain other neurotropic viruses have been shown to produce a regression of cancer.[34]

The whole spectrum of fundamental biological relationships involved in the complex virus-host relation has only just begun to be realized. When we consider the possibilities of pursuing such studies in germfree animals, where at least the factor of experience with other pathogens can be eliminated, the elucidation and clarification of some of these complex host-virus relationships may be somewhat simplified. Whatever response is obtained in the germfree animal can be considered to be a reaction between the agent introduced and the host tissue; presumably the results will be uncomplicated by the animal's previous experience (acquired resistance). The germfree animal has already been shown to be especially sensitive to conventional pathogens with which, from birth onward, the conventional animal lives in harmony and against which it develops defenses. There is no reason to expect that the germfree animal should not be more sensitive to viral infections than its conventional counterpart.

Studies in germfree animals could provide information relative to the question of proviruses or inapparent viruses. In fact, work reported elsewhere in this monograph by Reyniers and by Taylor *et al.* presents specific examples of just such a situation. At present it is difficult to conceive that a small quantity of MCA inoculated into an animal is anything other than an inciter or stimulus, but this small quantity of carcinogenic agent gives rise in the germfree chicken to a growth process that produces gross tumors and fibrosarcomas. From many of these tumors one may obtain extracts that contain large quantities of microsomal and other particles that for years we have been accustomed to call virus or viruslike. This becomes even more striking and curious when these particles are observable in increased quantity and remain and enhance their biological activity many passages removed from the original MCA stimulus. Microsomal particles have been associated with protein synthesis in cell metabolism, as they contain high proportions of RNA and phospholipid, both of which have been implicated as factors in carcinogenesis both by chemical carcinogens and the tumor-inducing viruses. The question "Where do viruses come from originally?" is indeed a difficult one, and one impossible to answer at present. Perhaps the germfree animal will assist us in making at least a beginning in this direction.

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Discussion of the Paper

Question: The statement has been made that the germfree animal is more sensitive to pathogenic agents than is the conventional animal. I have not seen documented evidence for this. At our laboratories we have attempted to induce serum hepatitis in 150 chicks, but none of these birds has exhibited anything resembling this disease; they were among the healthiest animals we have had. We have succeeded in inducing pleuropneumonialike organisms (PPLO) to grow in germfree chicks, and disease ensued at the same rate as in conventional chicks. I know of no reason, even a theoretical one, why germfree animals should be more susceptible to pathogenic organisms than are conventional animals. Wagner has shown that the immune response is the same in both cases.

Taylor: The questioner said that hepatitis did not develop in germfree animals, and then he said that another agent did develop, but at the same rates in germfree and conventional animals. By sensitivity I do not mean necessarily an increase in the rate of disease production. Because of limitations of time I omitted from my oral presentation several paragraphs, including a passage wherein I discussed this problem.

Briefly, as we work with germfree animals it becomes ever more apparent that previous experience with other pathogens can influence the response of an animal to individual experimentally introduced organisms, and this was the point that I was trying to make. It is possible that some of the information we have on viral diseases is faulty: for example, all things we now say are caused by viruses actually may not be caused by viruses; they may be caused by the interaction of entirely different pathogens in the host or by a virus in combination with these other agents.

T. D. Luckey (*University of Missouri Medical Center, Columbia, Mo*): I should like to call attention to the work of Cohendy *et al.* in 1922[1] in which it was shown that germfree guinea pigs would succumb to *Vibrio cholerae*, whereas conventional animals showed no response.

Reyniers (*Lobund Institute, University of Notre Dame, Notre Dame, Ind.*): In this connection let me mention a brief report made some time ago on serum hepatitis in germfree Leghorn chickens. The Akiba pool was the source of the serum, which was furnished by Henle of the University of Pennsylvania, Philadelphia, Pa. Conventional birds inoculated with this serum were negative, while the White Leghorn germfree chicks developed a fatal disease that could be transmitted to other germfree birds. We were unable to elicit any response rom either germfree or conventional New Hampshire Red chickens. We did ot claim this as an isolation of hepatitis virus, nor could we justify the use f the germfree (virus?) serum for further tests on human volunteers. We imply pointed out the difficulties inherent in using sera from conventional nimals or patients in a search of this kind because of the possibility of isolating ther viruses. Second, we called attention to the obvious fact that a species mmunity appeared to exist. We are pleased to hear of the results mentioned y Taylor. Until further investigations along this line are completed we have o other explanation to offer.

Another point that seems pertinent is that there are some bacteria that may e pathogenic in one host and not in another, so that the term pathogenic s relative. For example, we have recorded heart lesions caused by nonpathoenic bacteria and instances of *Sarcina lutea* being pathogenic in germfree nimals. *Bacillus subtilis*, which is not pathogenic to conventional animals, nay be definitely so to germfree animals of the same species. In collaboration vith my associate Morris Wagner I have shown that freshly isolated *Shigella ysenteriae* obtained from the Navy Medical Research Institute, Bethesda, Id., could be established in germfree animals at a level of 1 × 10^{-8} in 24 hours vhen administered orally. There was no visible effect within a 2-week observation period on the germfree rats or mice thus treated; that is, there were o ulcerative lesions or other adverse influence on the health of the animals. ttempts to establish such a flora in conventional animals at the levels we used ave been negative.

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REARING GERMFREE CESAREAN-BORN RATS, MICE, AND RABBITS THROUGH WEANING*

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Introduction

At the present time the method of choice for obtaining germfree rats, mice, and rabbits is the hand feeding of sterilized diets to cesarean-born young. Alternative possibilities have not yet proved satisfactory. Complete decontamination of conventional adult animals has not been reported. Cross-suckling cesarean-born young on germfree foster mothers of other species might create problems for the virologist, oncologist, immunologist, or biological purist. A third type of alternative to hand rearing might be found in the work of Rowntree,[1] who injected thymus extract into rats for 9 generations. By the tenth generation his rats were born with their ears open and their incisors erupted. At 48 hours, they had their eyes open and were able to eat solid food. Rowntree himself found the method difficult to duplicate, and no one has yet tried it for obtaining germfree animals.

The hand rearing of germfree mammals was first reported for such species as the guinea pig[2] and the goat,[3] whose stage of development at birth is relatively close to that of the weaned animal. The hand rearing of rats and mice, which are much more helpless at birth, remained an unexplored possibility until much later. Obtaining germfree colonies of these species has therefore required not only pioneering in the germfree phase of the work, but also an original investigation into the entire technique of hand rearing.

The hand rearing of rats was first reported by Gustafsson in 1946.[4] The first detailed report on the technique involved was published by Reyniers *et al.* in Lobund Report No. 1[5] later in the same year. Gustafsson gave the details of his method in 1948 in "Germfree Rearing of Rats."[6] At Lobund Institute the rats were offered, at hourly intervals, an opportunity to suck autoclaved milk formula from a rubber nipple. In the method of Gustafsson, a thin rubber tube was inserted halfway down the esophagus, and sterilized milk formula, including some predigested casein, was forced into the stomach gradually over a period of several minutes. The stomach could thus be well filled and the feeding interval prolonged to 4 to 5 hours.

Since the publication of Lobund Report No. 1 a different technique of germfree rat rearing has been tested at Lobund Institute with more consistently reproducible results than the method originally reported. The new technique involved forced feeding with a short, tapered nipple and the use of a relatively simple milk formula. This new technique then proved successful in weaning, for the first time, Swiss albino and C3H mice. After earlier preliminary experiments, germfree rabbits were successfully hand-reared at Lobund Institute

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in the years 1949 to 1952 by Luckey and Teah.[7] Germfree rabbits are now being raised at Lobund Institute on different types of diet.

The method of rat rearing described in Lobund Report No. 1 had weaned more than 200 rats and had produced an extensive rat colony, but the hand-rearing results were not consistently reproducible. The approach followed at that time might be described as the imitation of nature, an attempt to duplicate, as far as practicably possible, the environmental conditions, composition of diet, manner and schedule of feeding, and other details of normal maternal care. In line with this approach, the young were fed by nipple and were given a complex artificial milk formula designed to imitate rat's milk. Rat's milk itself was not used, because of the practical difficulty of obtaining it and because it did not remain fluid after steam sterilization.

The difficulty with nipple feeding was that an intake of diet adequate in quantity for steady growth could not be assured. This fact was demonstrated by observations of T. J. Mende while at Lobund Institute (personal communication).

The possible problem with feeding this very complex artificial diet, which resembled rat milk but did not imitate it perfectly, was that the individual components of the diet within this complex might have very different effects from those of their natural rat milk counterparts. Various types of toxicity, imbalance, and indigestibility might occur and be difficult to trace. Since these difficulties of diet and intake might be responsible for the inconsistent results obtained with hand-reared rats at that time, a new line of approach was adopted in 1953.

A very simple forced-feeding technique that could be mastered by a crew of inexperienced feeding technicians was developed. A diet was chosen for its ease of feeding and for its relative simplicity, rather than for its theoretical adequacy. When it was found that rats could be weaned by this procedure the policy was adopted of adding to this diet only such other components as could prove their particular value and their compatibility with generally good rearing results. Vitamin supplements were studied first.

In the course of following this policy, a point was reached at which results were adequate to provide weaned hand-reared rats virtually at will, and several new colonies of germfree rats were started. Even though at that point the diets were still obviously inadequate for optimal growth, further germfree hand rearing of rats was discontinued in order to free the germfree equipment for the rearing of new species. Changes in the diets were then developed through experiments with contaminated hand-fed rats, as indicated by Wostmann elsewhere in this monograph. These changes eventually lead to formula L-449C, a diet more closely resembling the composition of natural rat milk. The techniques and diets reported here are those actually used in starting and maintaining our present colonies of germfree rats and mice, even though this was accomplished at a point in our diet development program when a number of obvious deficiencies, notably of protein and minerals, had not yet been investigated. More recent and more adequate diets are being used in our current program of raising germfree rabbits.

Experimental Technique

Rats and mice. Two important improvements in the cesarean operation have been introduced recently by J. A. Reyniers.[8] These are, first, delaying the operation until one baby has been delivered spontaneously by the mother, and second, elimination of the previously employed seconal anesthesia. With rats, a fatal or near-fatal blow was delivered at the base of the mother's skull just before the operation. With mice 2 per cent novocaine was injected along the midline, following a technique developed by Bruce P. Phillips for operations with guinea pigs (personal communication).

Within the Reyniers Germfree Isolator[5] the young were kept all together inside a small rearing pan having a cloth floor. The temperature inside this pan was maintained (±2°) at 35° C. for the first 4 days, then dropped by 1° C. every 4 days until the young were weaned at 25 days. The animals achieved some variation in temperature by huddling together or scattering. Additional moisture within the warmed rearing pan was provided by strips of wet cloth.

Forced-feeding technique. A tapered rubber tube was inserted just into the upper esophagus while the animal was actively swallowing. Diet was then forced through the tube into the stomach from a calibrated syringe. Because of its taper, the tube tended to block the opening of the esophagus against regurgitation. Since the tube also tended to block the respiration of the animal, it was necessary to force the diet into the stomach within a few seconds after insertion of the tube. The size and shape of rat- and mouse-feeding tubes are shown in FIGURE 1. They were made of natural-rubber latex, as described in Lobund Report No. 1[5].

The first 2 or 3 feedings after birth were given at hourly intervals and consisted of sugar-free Tyrode's solution. Succeeding feedings were of milk formula and were made at 2-hour intervals. During the first week the rats were fed diet L-420, which consists of equal parts of cow's milk and light cream. with the addition of a proprietary vitamin mixture for infant feeding, and vita-

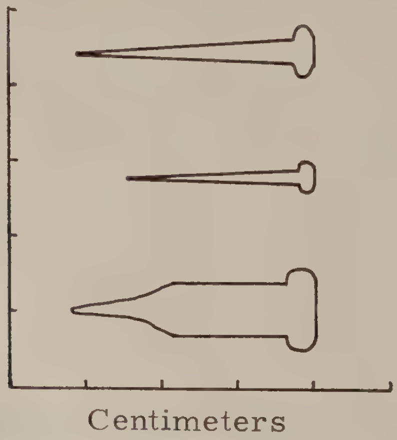

FIGURE 1. Shape and actual size of the rubber feeding tubes used for hand feeding: top, for rats; center, for mice; bottom, for rabbits.

TABLE 1
MILK FORMULAS AS PREPARED FOR AUTOCLAVING

	420	449C	449G-E1
Cow's milk, whole	50 ml.	50 ml.	25 ml.
Light cream (18 per cent)	50 ml.	50 ml.	75 ml.
Vi-Syneral*	0.1 ml.	0.2 ml.	0.2 ml.
Mixed tocopherols (253 I.U. vitamin E per gram)	1.7 mg.	3.3 mg.	6.6 mg.
Vitamin K (menadione)	0.17 mg.	0.33 mg.	0.33 mg.
dl-Methionine		0.2 gm.	0.2 gm.
l-Tryptophan		0.06 gm.	0.06 gm.
Skim milk protein†		4.0 gm.	6.0 gm.
l (−)-Cystine			0.1 gm.
Choline dihydrogen citrate			0.1 gm.

* Vi-Syneral aqueous vitamin drops (U. S. Vitamin Corp., New York, N. Y.) is listed on the label as containing the following per 0.6 ml.: vitamin A (natural), 5000 U.S.P. units; vitamin D (natural), 1000 U.S.P. units; ascorbic acid, 50.0 mg.; thiamine HCl, 1.0 mg.; riboflavin, 0.4 mg.; pyridoxine HCl, 0.3 mg.; niacinamide, 5.0 mg.; pantothenic acid, 2.0 mg. (made soluble by sorethytan esters).

† Skim milk dialyzed against KCl, NaH_2PO_4, Na_2HPO_4 solution for 24 hours, then freeze dried.

mins E and K (TABLE 1). After about one week, the proportion of butterfat in the diet, originally 10 8 per cent, was reduced to 7.2 per cent by combining only one part cream with three parts milk. The diet was ampuled and sterilized into the germfree isolator in 121° C. steam for 15 min.

The mouse diets were exactly the same, with the addition of 0.05 per cent quercetin* added as a source of vitamin P. It was hoped that this supplement might reduce the incidence of lung hemorrhage by its effect on capillary fragility.[9] There was no opportunity to prove the effectiveness of this supplement by controlled experiment.

Salts of copper, iron, and manganese were sterilized in a separate solution, along with zinc, which is known to be much higher in rat's milk than in cow's milk[10] and has been shown to be a colostrum factor for mice.[11] A general mixture of purified B vitamins was also sterilized separately. These mineral and vitamin supplements (TABLE 2) were added to the milk formula inside the germfree isolator. Vitamin C solution (4 mg./ml.) was fed in place of all or part of a milk feeding at 12-hour intervals in view of the indications for its use reported in Lobund Report No. 1.[5]

The amount of this diet fed to rats on the first day was about 0.1 ml. per feeding, increased by 0.05 ml. every 2 to 3 days. For mice the starting amount was 0.04 ml. increased by 0.01 ml. every day, but the schedule was not followed rigidly.

The rats or mice were stimulated for urination and defecation after each feeding by lightly stroking the perineal region with a piece of sponge rubber. At 2 to 4 days of age the mice sometimes required pressure on the abdomen during stimulation to bring about full release of urine.

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* Provided by F. J. Kirchmeyer of Abbott Laboratories, North Chicago, Ill.

TABLE 2

VITAMIN AND MINERAL SUPPLEMENTS ADDED AFTER SEPARATE STERILIZATION TO MILK FORMULAS FOR VARIOUS SPECIES

(ml. per 100 ml. Original Milk Formula)

	Rat	Mouse	Rabbit
B vitamin mixture 103*	1.2	3.0	3.0
Four tenths per cent ascorbic acid	9.0	9.0	
One per cent calcium ascorbate			5.0
Salts 13†	0.6	0.5	
Salt Solution 15‡			2.0
Ten per cent calcium gluconate§		5.0	
Eight per cent $CaCO_3$ suspension			3.0

* B vitamin mixture 103 contains the following in 3 ml. of water solution: 3 mg. thiamine HCl; 1 mg. riboflavin; 1 mg. pyridoxine HCl; 2.5 mg. niacinamide; 12.5 mg. calcium pantothenate; 100 mg. choline dihydrogen citrate; 0.025 mg. biotin; 0.25 mg. folic acid; 0.025 mg. vitamin B_{12}; 100 mg. inositol.

† Salts 13 contains the following per ml. of water solution: 53.4 mg. ferric citrate, U.S.P. VIII; 6.4 mg. $CuSO_4 \cdot 5H_2O$; 9.0 mg. $MnSO_4 \cdot H_2O$; 10.7 mg. $ZnCl_2$.

‡ Salts 15 is made up of equal parts solutions A and B, sterilized separately. Solution A (per ml.): 176 mg. KH_2PO_4; 180 mg. Na_2HPO_4; 0.4 mg. KI. Solution B (per ml.): 14 mg. $MgSO_4$; 4 mg. $MnCl_2 \cdot 4H_2O$; 24 mg. ferric ammonium citrate; 2.4 mg. $CuCl_2$; 5.2 mg. $ZnSO_4 \cdot H_2O$; 0.8 mg. $CoCl_2 \cdot 6H_2O$.

§ Abbott's calcium gluconate-glucoheptonate.

At 15 days of age, both forced feeding and stimulation could be discontinued, and milk formula was thereafter offered in a small dish along with other dishes of water and solid food. By 25 days the young were weaned to solid diet and water.

Rabbits. The technique for rearing germfree rabbits resembled closely the earlier rat-rearing technique described in Lobund Report No. 1.[5] Because of a lack of sufficient pregnant animals, the cesarean operation was performed without waiting for spontaneous delivery to begin. When only a few animals are available, all of these might deliver at very inconvenient hours or might deliver so rapidly that they could not be operated on in time. Local anesthesia was used, as for mice. Operation at 31 days was considered preferable, when possible, to operation at 30 days' gestation. The pregnant rabbits were of the larger breeds and generally of mixed ancestry.

After the first several days, the environmental temperature was kept 1° to 2° C. lower than the usual temperature for rats and mice. The rabbits were allowed to suck every 2 hours from a soft rubber nipple (FIGURE 1) attached to a calibrated 10-ml. dropper with a rubber bulb. Except during the first day or half day, the rabbits tended to take too much diet rather than too little, and the feeding technician usually had to limit their intake. The rate of flow could be controlled by the size of the nipple opening, but adjustment was difficult with the thick milk formula used for rabbit feeding.

The diet L-449G-E1 (TABLE 1), while still based on cow's milk and cream, contained additional dialyzed, freeze-dried skim milk protein, plus cystine and choline, this combination having been found to eliminate abnormally fatty livers in the hand-reared rabbits.[12] The rabbits received a more complete mineral supplement than that used for rats and mice (TABLE 2). The amount ed was gradually increased from 1 ml. per feeding on the first day to 5 to 6 nl. per feeding on the twenty-first day.

Stimulation to induce urination was carried out as for rats and mice, but lefecation usually occurred spontaneously even in the early days of life. The abbits were weaned to dish feeding of the milk formula at 21 days, with water nd solid food also available, and were weaned to solid food at 28 days.

Results

Rats. The immediate result of adopting the forced-feeding technique was onsistent reproducibility of results. Rats were weaned in 18 of the following 25 experiments, whereas rats had been weaned in only one of the preceding 25 xperiments using the technique discussed in Lobund Report No. 1.[5] The echnique proved easy for the feeding technicians to learn and eliminated much f the personal factor in hand-rearing success.

The forced feeding of even relatively simple diets was successful in weaning nost of the rats from any litter that was full term and not unusually low in verage birth weight. On the simplest diets, however, before all the vitamin upplements had been tested, there was a high incidence of leg deformities, xtreme cecal enlargement, and infertility. Changes in vitamin supplements, n diet proportions, in operative technique and feeding schedules were made ntil the procedure outlined above was evolved. Following adoption of this ull procedure, 85 per cent of the young started in 5 experiments were weaned 77/91), and the only deaths during the feeding period were due to accidental auses: aspiration pneumonia, and rupture of esophagus, intestines, or pharyngeal mucosa. Nearly all females weaned proved fertile, while leg deformity nd extreme cecal enlargement were reduced to minor proportions.

FIGURE 2 shows that the rate of gain, although very low, was steady in ontrast to the dip in weight that occurred in earlier experiments when rats vere fed by nipple. Sporadic attempts to increase growth by more rapid ncreases in food intake appeared to be responsible for an increase in accidental leaths and were abandoned. Rats of the Holtzman, Harlan, Long-Evans, nd Lobund (inbred Wistar) strains were reared.

As in earlier rat-rearing experiments, all rats showed cataracts and lost nuch of their first hair at 3 to 4 weeks of age, regaining a full coat at 4 to 5 veeks. Later test experiments, however, showed that cataracts could be liminated, and hair retention moderately improved by adding 0.1 per cent nethionine and 0.03 per cent tryptophan to the liquid diet, even if nothing urther was done to improve its obviously low protein content.

Swiss mice. The response of Swiss mice to the hand-rearing technique was lmost identical with that of rats, with one serious exception. The mice were ar more susceptible to feeding and handling injuries, especially when fed enough iet for steady weight gain. In 10 experiments using the full technique decribed above, 6 per cent of the mice (8/134) were weaned. Cataracts and leg eformities were not noted, but a rachitic rosary was often noticeable. This st-named condition was not unexpected on a diet to which, at this stage of xperimentation, no supplements of Ca or P had been added.

C3H mice proved even more difficult to rear. On the basal rat diet with

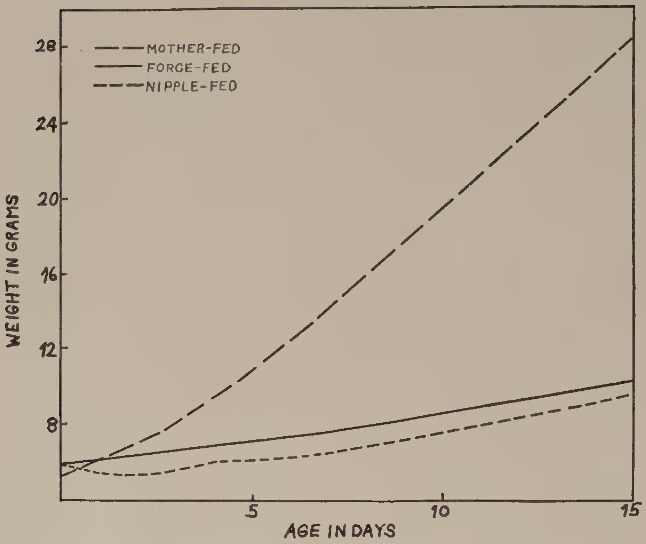

FIGURE 2. The growth of germfree rats according to the method of feeding. Each curve represents a composite of the more typical experiments in each category.

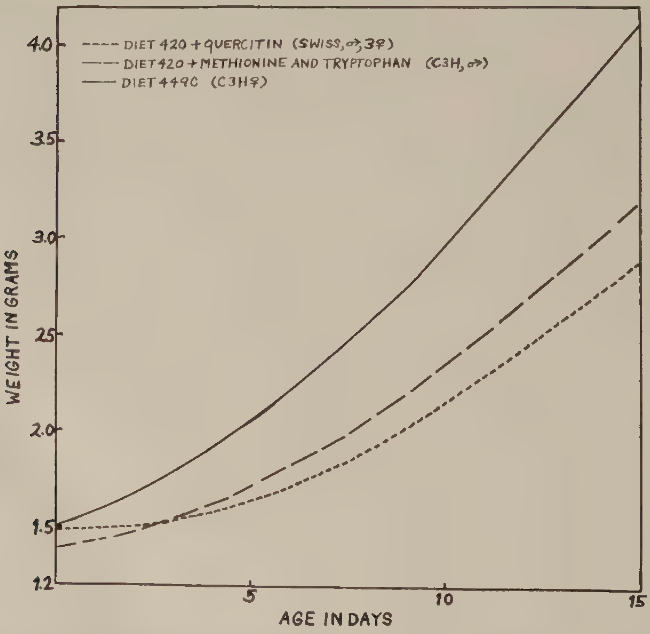

FIGURE 3. The growth of hand-fed germfree mice on 3 different sterilized diets.

lded methionine and tryptophan, only 1 male of 95 young was weaned. A ide variety of dietary changes was then gradually introduced, following leads ken from pilot experiments on contaminated rats and mice. One female as eventually weaned on diet L-449C (TABLE 1) with 0.2 per cent choline Cl lded, and with 1 per cent Salt Solution 15 in place of Salts 13. Superior owth on this diet is shown in FIGURE 3. Fifteen subsequent attempts to ar C3H mice were unsuccessful, although cesarean-born C3H mice could sily be reared by germfree Swiss mice as foster mothers. The chief cause loss in hand-fed C3H mice was not obvious mechanical injury, but a syn- ome of pulmonary hemorrhage and edema, without clear-cut inflammatory anges, occurring almost exclusively on the fifth and sixth days of life. Owing accidental contamination, the hand-reared C3H mice did not have an op- rtunity to reproduce.

The weaning of rats and mice from hand feeding at 15 days, instead of the 21 25 days mentioned in earlier reports,[5, 6] resulted in improved growth and fety for the animals and a saving in work for the feeding staff. The only rly postweaning deaths were due to intestinal volvulus, apparently associated ith cecal enlargement.

Rabbits. Rabbits have been successfully weaned in 13 of 17 germfree ex- eriments on diet L-449G-E1 (TABLE 1, with supplement shown in TABLE 2). he weight gain (FIGURE 4) was much closer to normal than that for hand- ared mice and rats. Twenty-three per cent of the germfree young started

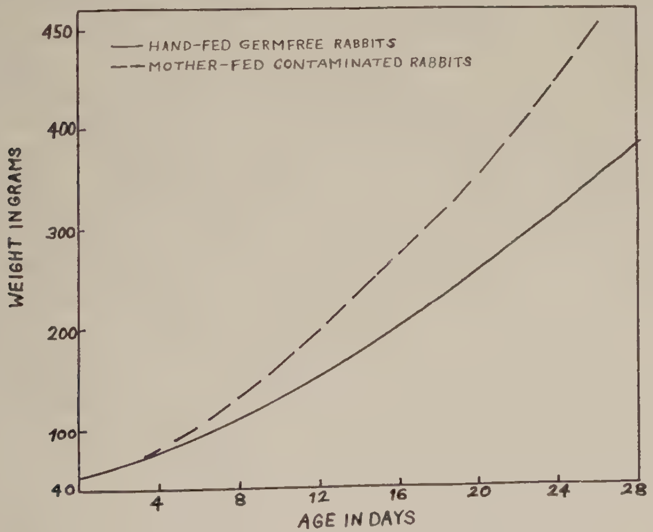

FIGURE 4. Growth curves of hand-fed germfree rabbits and of mother-fed conventional bbits. A composite curve has been made from the more typical germfree experiments be- use the animals used were from mixed strains having varying growth potentials. The ve for the mother-fed conventional rabbits has been taken from Hills and MacDonald,[16] o have been able to hand-rear conventional rabbits at a rate of gain similar to that for d-reared germfree rabbits at the Lobund Institute.

(30 of 130) were weaned. The losses were almost entirely due to a sterile aspiration pneumonia or to a sudden occlusion of the respiratory passages by lumps of dietary material. These losses tended to be grouped together and to occur at the third to sixth days. The incidence of such losses could be much reduced by a drastic lowering of the food intake, but the resulting prolonged inanition prevented successful weaning to solid diet.

Hair loss has been a matter of individual occurrence. Within a group it usually occurred in those individuals making the slowest weight gain. The bones have tended to be fragile; ribs and femurs have occasionally been found broken, presumably by rough handling or by the rabbits' own activity, but ossification appears to be retarded rather than disorganized.

In contrast to rats and mice, rabbits are subject to serious losses during the postweaning period from extreme cecal enlargement, intussusception, nephrosis, and causes still unknown. Determining the relationship of the hand-feeding regimen to these occurrences must wait for further experimentation and for the obtaining of second-generation germfree rabbits.

Discussion

The fact that germfree rats, Swiss mice, and rabbits have been successfully hand-reared on rather simple practical formulas is a welcome indication that success does not depend upon any special factors peculiar to these species or to the germfree state. In fact, hand rearing under germfree conditions has consistently proved more successful than hand rearing under contaminated conditions (unpublished data). Efforts to obtain optimal growth and development might reveal special requirements, but for simple weaning the limiting factor so far appears to be quantitative rather than qualitative.

With the techniques described, the intake of food is now limited, not by the animal's willingness to eat, but by its ability to pass the food through the intestinal tract. Even though growth has been definitely improved by addition of protein to the diet, the intake possible on this type of diet is still low.

Low intake of food thus appears to be the primary cause of retarded growth. Other disabilities such as hair loss and retarded skeletal development, usually more conspicuous in the slower growing individuals, may likewise reflect low intake rather than specific deficiencies, although these are not excluded.

Mortality among hand-reared animals, except C3H mice, appears to have been chiefly the result of feeding more diet than the animal could handle. This was clear enough when there was rupture of some part of the digestive tract or a great accumulation of undigested food, but the evidence also points to overfeeding as the cause of most cases of aspiration pneumonia.

For rats there was a wide margin of safety between what we might call the minimum effective dose of diet (the smallest amount needed for steady weight gain to eventual weaning) and the lethal dose of diet, causing mechanical injury to the digestive or respiratory tracts. For rabbits there was a less wide margin of safety, and for Swiss mice a very narrow margin of safety.

C3H mice, however, were lost chiefly through a special pulmonary syndrome whose relation to this dilemma of underfeeding and overfeeding is not clear, unless the syndrome should prove to be the result of low serum protein. The noninflammatory pulmonary edema and massive hemorrhage seen so often at 5 to 6 days might well be a sequel to neonatal atelectasis which, in turn, could result from inadequate stimulation of respiration following cesarean birth. On the other hand, a grossly similar syndrome is sometimes seen in hand-reared mice that have been born naturally by conventional stock mothers. Furthermore, cesarean-born C3H mice are readily reared by germfree foster mothers of the Swiss strain. It is thus not yet clear to what extent cesarean birth is responsible for the respiratory changes. In view of its possible influence, however, various stimuli to the respiration of cesarean-born young, such as increasing the CO_2 content of the atmosphere, pressing the placenta before the cord is cut,[13] and extra handling of the animal, have been considered but not yet attempted.

The lung syndrome closely resembled the "neonatal cold injury" recently described for human infants.[14] Experiments are planned to see if our standard rearing temperatures are too low for hand-reared C3H mice. These animals are known to have a high caloric requirement, and the low food intake presently possible in hand rearing may handicap them in maintaining body temperatures during the first week of life.

Improvements in the safety of hand rearing and in the condition of the young are being explored from two directions. On the side of feeding technique itself, the effect of speed of intake on the digestive process and on the safety of feeding is being tested. Gustafsson's technique,[6] which permits slower injection of diet, has proved difficult to adapt to mouse feeding by our feeding staff. We are now testing, with contaminated hand-reared rats, ways of achieving greater intake with slow nipple feeding than was previously possible.

Sometimes the problem of intake has been aggravated by an accumulation of air in the stomach, which has occurred during both nipple feeding and forced feeding. While neat and careful feeding may be the decisive factor here, it is possible that the fluid consistency of the stomach chyme formed by hand-fed diets may help to keep air trapped in the stomach. A diet is being tested in which the casein, concentrated by centrifugation, tends to form a firm, layered curd in the stomach. Also, under certain conditions, a partial blockage of the throat by dietary material may cause diversion of respired air down the esophagus into the stomach. Similar occurrences have been observed by Gustafsson.[6]

With feeding techniques of all types attempted thus far there seems to be a natural limit to the amount of food that the animal can digest, and this amount s probably determined by the character of the diet. One of the most promisng avenues of investigation appears to lie in the use of completely water-soluble, low-residue diets such as have been recently developed.[15] Diets made partly of protein hydrolyzates have been used by Gustafsson,[6] but our experience with them was not encouraging. Completely soluble diets, however, f they can be satisfactorily sterilized, are being considered for trial, as they would impose less burden on the digestive ability of the gut, would cause less abdominal pressure, and would have the special advantage of relative freedom rom dead bacteria and their possible antigenic effects.

While much progress toward adequate intake can undoubtedly be accomplished by empirical tests, the ultimate basis for an optimally successful hand-rearing program will probably be an understanding of the physiology of the suckling animal, and the reasons for its responses to hand-rearing diets and techniques.

Summary

Germfree rats, mice, and rabbits have been successfully weaned from hand rearing by relatively simple procedures.

The rate of success was high for rats, moderate for rabbits, and low for Swiss mice. These differences appeared to reflect differing margins of safety between the amounts of diet required for growth to weaning, and the amounts that could be fed with little risk of injury to the digestive or respiratory tracts.

Although growth and development were subnormal during the suckling period, the diet appeared to be sufficiently balanced in its insufficiencies to create few cases of permanent handicap.

While much can still be done in an empirical way to improve intake, growth, and development, optimal results may demand a thorough understanding of the physiology of digestion in the suckling animal.

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NUTRITION AND BIOCHEMISTRY OF GERMFREE CHICKS*

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Very few investigators have examined the biochemistry of germfree animals. Except for contributors to this monograph, only Küster[1] presents any analytical biochemistry in his writings. However, we may read biochemical significance into most of the experiments performed throughout the history of germfree research.

The operations involved in obtaining and maintaining a pure culture of bacteria are similar in principle to those needed for obtaining and rearing germfree animals. One is done on a microscale with small apparatus, the other on a macroscale with large apparatus; the two are linked both historically and technically by work with germfree plants. Early workers in legume physiology were concerned with the biosynthetic abilities of the plants involved. They found it necessary to isolate the plants completely from contaminating or indigenous microorganisms before they could begin to understand the biochemistry of the interrelationships between the plant and microbial symbiants involved. Duclaux's investigation into the nitrogen metabolism of peas[2] led Pasteur to propose germfree experimentation. Based upon preconceived ideas of the contribution of the intestinal microorganism to the nutrition of the host, Pasteur[3] suggested that life without bacteria would be impossible. Nencki's answer[4] was also based upon a biochemical consideration. He suggested that life without microorganisms might be easier, since bacteria produce indole, skatole, and other toxins. Thus we see that the first thoughts of rearing germfree animals were steeped in biochemistry.

Nutrition has had a direct and more practical role in the development of germfree research. One might suggest that many experiments with germfree animals resulted in a failure due to lack of knowledge of the principles of nutrition as we know them today. Thus the pioneer experiments of Nuttall and Thierfelder,[5] who tried to rear guinea pigs in a bell jar system, failed because of apparent nutritional deficiency. One could also suggest that the failures of Schottelius[6] to rear germfree chicks resulted from nutritional failure. Schottelius concluded that life without bacteria is impossible. The turning point came in 1912 when Cohendy[7] found that he could raise germfree chicks satisfactorily. The varied diets he used were apparently more nearly adequate than those used by his predecessors. Küster maintained germfree goats satisfactorily for a short period of time.[1] In his limited experiments he obtained some data regarding the nitrogen excretion and dry weight of a few tissues of goats in the germfree state. In 1913, Wollman[8] found germfree tadpoles could be reared if they were fed properly. Guyenot[9] indicated that meat fly larvae grow much better when yeast is added to the diet. In subsequent years he found that yeast extract also could be substituted for the yeast.

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His finding that germfree flies live longer than control flies may indicate the sagacity of Nencki.

The nutritional problems of the guinea pig, however, still defied investigators. In 1914 Cohendy and Wollman[10] found they could not grow guinea pigs satisfactorily in the germfree state. In 1922 the same investigators[11] again found that germfree guinea pigs grew poorly. They did note, incidently, that a single contamination of *Vibrio cholerae* could be maintained in germfree guinea pigs while it was lost in the contaminated guinea pigs. The guinea pigs of Glimstedt,[12] reported during the 1930s, grew slowly and did not appear to be without nutritional problems during the one to two months they were maintained. Glimstedt's finding of marked reduction of lymphatic centers and reduction in the white blood cell count in tissues and blood is, in the final analysis, an indication of a nutritional or biochemical lesion. Something was not stimulated properly, or some compound was missing for proper function of these defense mechanisms.

Some of the early work has not been reported adequately to evaluate results even in these general terms. This is true with the rearing of germfree dogs by Glimstedt[13] during the 1940s and the early work of Reyniers and Trexler[14] with guinea pigs, rabbits, chickens, mice, and rats. However, germfree monkeys appeared to grow satisfactorily. Balzam[15] found germfree chicks could grow at about the same rate as conventional chicks fed the same sterilized diet. Bacterial contamination did not appear to ease the vitamin B deficiency produced. In 1946 both Reyniers *et al.*[16] and Gustafsson[17] report work with germfree rats. Subsequently the author found that, given any one of a variety of proper diets, germfree rats grow satisfactorily and reproduce. Unfortunately time and the topic assigned do not allow presentation here of details of the nutritional problems encountered in producing the first colony of germfree rats.

The work presented in this paper summarizes most of the essential data on chickens that I have obtained over a period of many years. Some of it has been published with coauthors, and some of it will be presented as a comprehensive survey of the germfree chicks that is presently in press. It should be realized that the number of animals available limited considerably the approach and scope of this work. In such work more is done outside of the germfree cages than is done inside them. In other words, before a sterilized diet is tried in germfree conditions it is tested extensively in conventional animals. Thus one or more generations are often used before trying the diet on monobiotic animals. These diets necessarily have an excess of vitamins and are high in proteins. The nutrition survey was done to cover as much territory as possible in as short a time as possible. Consequently, deficiencies were not studied as thoroughly as possible; the approach was rather to survey the qualitative requirements of germfree chicks to see if they were similar to those of conventional birds. However, it will be seen that the analysis of those chicks will allow certain interrelationships in vitamin metabolism to be suggested.

Methods

In view of the interest shown in the instrumentation for rearing germfree vertebrates, it seems reasonable to illustrate the types of cages in which we

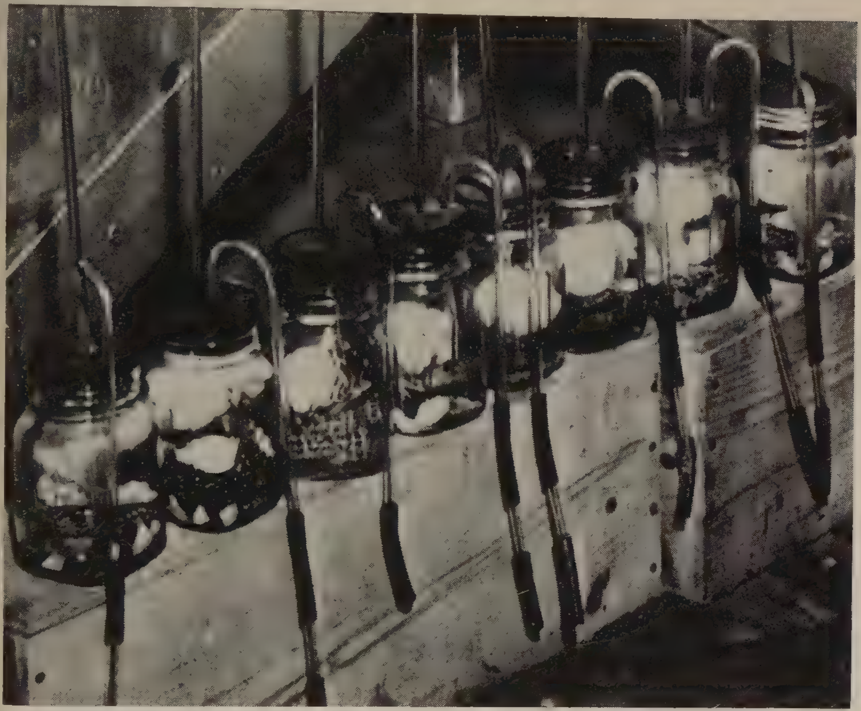

FIGURE 1. Simplified apparatus for rearing germfree chicks (University of Wisconsin, Madison, Wis.).

have obtained germfree chicks. In general, they will seem to run a gamut from little more than a test tube to a large complex apparatus. In principle, they have a common denominator with the use of the culture tube in bacteriology: there is a complete physical separation between the usual contaminated environment and that environment that has been presterilized for the germfree animal. The separation material may be of glass, steel, metal, rubber, or even a filter and sometimes is not more than air flowing under pressure. FIGURE 1 shows a rather simple apparatus, actually a pint Mason jar, that was used to hatch chicks and to maintain them for one to three days for various purposes. The filtered sterile air comes into the apparatus through the cover. In this case the air filter was simply what a bacteriologist would use in those days—a plumber's pipe with a nipple placed at each end and filled with cotton or a combination of cotton and glass wool. Sterile media may be admitted through the air inlet by a connection to the manifold of the filtered air system. The nutrient media is poured over the chicks and falls to the bottom of the cage, where it picks up any organisms from the air or droppings or feathers of the chicks. The cage floor itself is the bacterial test medium. The chicks are on a three-eighths-inch screen raised above the liquid, and the ratio of air flow to the size of the cage is sufficiently high to prevent excess humidity from becoming a problem. Contamination can be observed directly through this glass jar. The inside tube of the air outlet is designed in such a way that it can be pushed into the medium by outside manipulation, and the air pressure from above delivers one or more drops of the fluid aseptically onto the microscope slide or into another media for further incubation. The outside tube of the air outlet has a special connection for this purpose. The rubber keeps the glass tip sterile. The air outlet was attached to a manometer and the air pressure and the air flow in the cage could be adjusted by the size of the orifice of the manometer. The system was indeed simple and not the most useful in the world, but it did deliver germfree chicks routinely for various purposes when they were needed only a short period of time. Canning jars with nutrient media were also used to test for sterility by placing the chick inside *in toto*.

FIGURE 2 illustrates another simple apparatus that can maintain chicks for a month with no difficulty. Chicks in these churn jars are useful for investigations into vitamin deficiencies that develop within a few weeks. The food

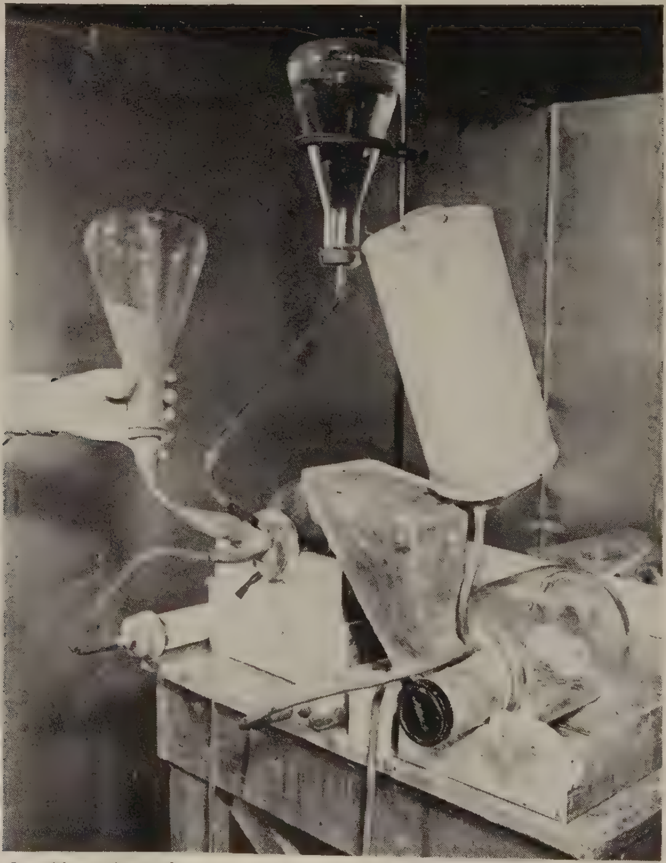

FIGURE 2. Churn jars adapted for rearing germfree chicks (University of Wisconsin).

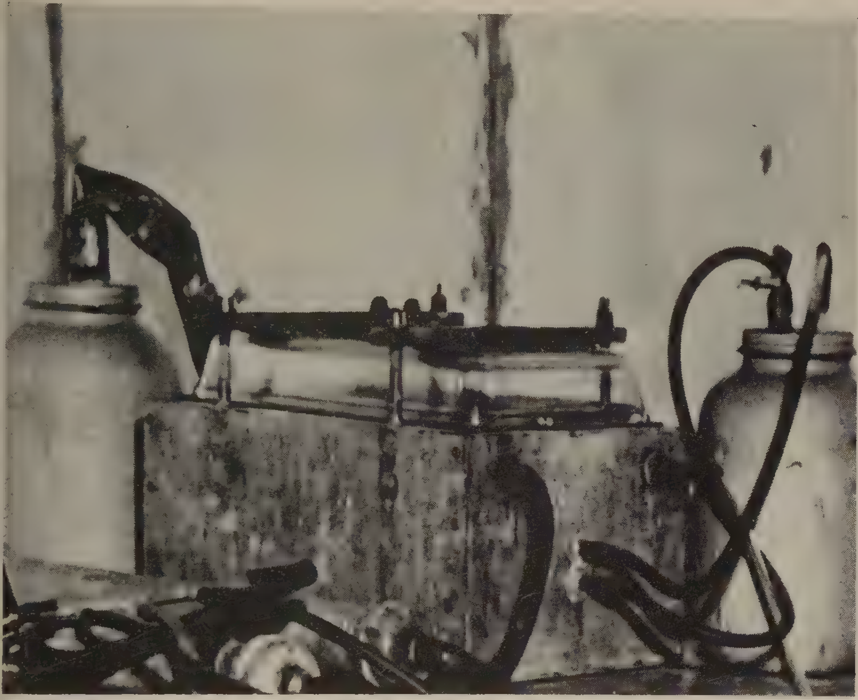

FIGURE 3. Simplified germfree apparatus (University of Wisconsin).

and water on some models is sterilized in place and, in other models, it is sterilized in separate containers. As can be seen on the far jar, the sterile food is "milked" into place and the sterile water is simply poured into the system. Another type of cage, also used early in the germfree work at the University of Wisconsin, is shown in FIGURE 3; this metal cage represents a very useful type since many things can be attached to it for different purposes. The food is "milked" through a 1-inch hose, and the water is delivered from the water cage, which has one tube for water flow and another for replacement air from the germfree cage. Another interesting aspect of this apparatus is that the outlet air simply goes through a long (about 18-inch) glass tube pointed downward. The diameter of the opening of this tube controlled the pressure and air flow of the cage. This tube was similar to the system used by Pasteur in which the neck of a flask was pulled into a small tube and kept open to show that gas may be exchanged with air without eliciting spontaneous generation. The difference is that the tubes on the chick cages have air flow under pressure. The type of chamber used in early work at the University of Wisconsin to maintain a clean environment around the germfree apparatus is shown in FIGURE 4. The apparatus itself was at this time essentially a large battery jar inverted with the open edge submerged in a germicidal solution.[18] This was found to be so humid that it was not too satisfactory. A small metal

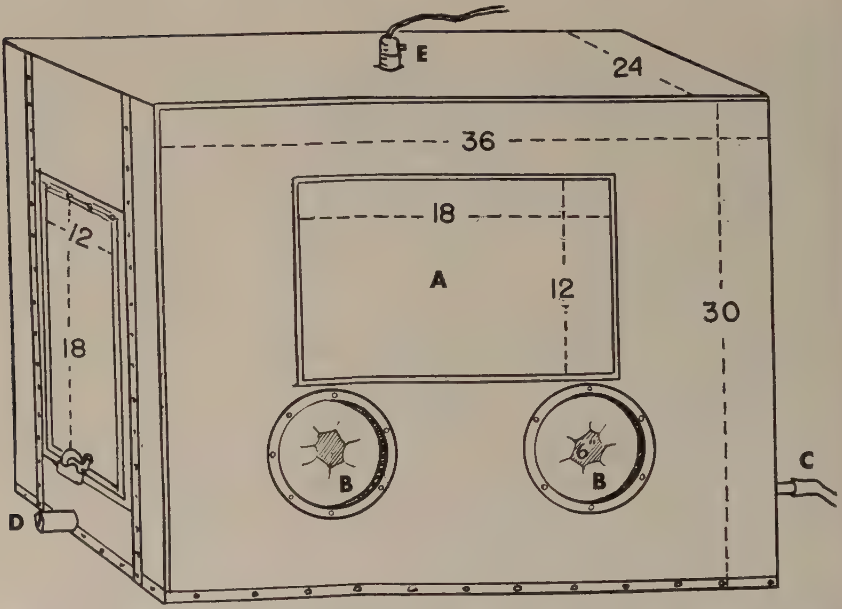

Figure 4. Sterile chamber, adapted from Hopkins *et al.*[19] *A*, glass viewing panel; *B*, glove ports; *C*, air inlet; *D*, outlet; *E*, electric light. Dimensions shown in inches.

cage similar to that shown in figure 3 was used within the chamber. The chamber was copied from the cages used by early workers in nitrogen fixation of legume plants.[19] Another simple cage, shown in figure 5 was developed at the University of Notre Dame, Notre Dame, Ind. While it seemed to work satisfactorily, it was never used extensively. It will be noted that the hatching deck keeps most of the germicide-containing eggshells from the chick as soon as the chick falls from the deck. The water container carries its own filter for replacement air. Both food and eggs are added through the large (stoppered) hole in the cast aluminum top. All of these simplified cages were sterilized in an autoclave.

Figure 6 illustrates the interior of the RGF series No. 50 cages in which chickens were maintained in two groups to compare their growth requirements, using two separate diets. Most of the work with chickens reported here was done with such apparatus, which has been adequately described by Reyniers *et al.*[20] Finally, it may be worthwhile to illustrate the room being prepared for germfree chicks at the University of Missouri (figure 7), which follows principles of Schottelius. One should have a roughing-out process and not try to maintain strict sterility separate from a contaminated environment with one barrier, but rather maintain a germfree apparatus in an ultraclean room with practically no contamination. In practice, one is amazed at the number and extent of leaks that can develop in a germfree system while sterility remains unbroken. It is suggested that if these cages are maintained in an ultraclean room the number of contaminations due to leaks in the system will be decreased.

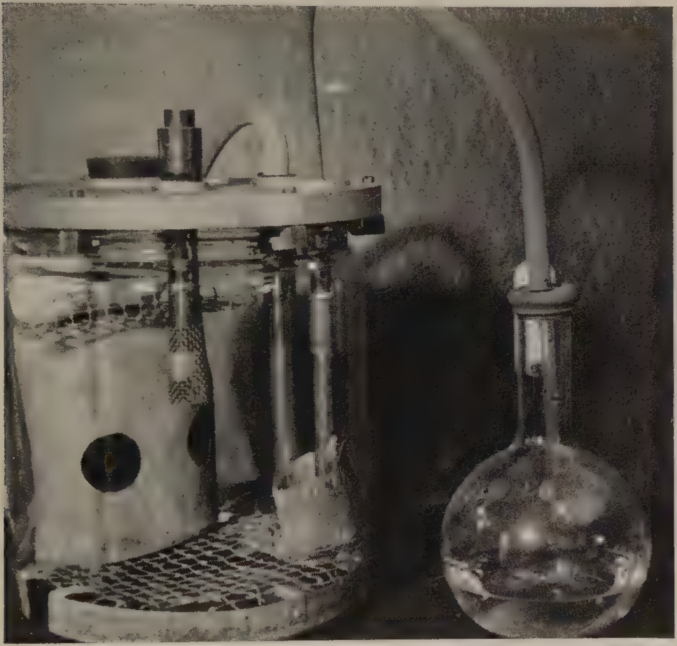

FIGURE 5. Simplified apparatus for rearing germfree chicks (University of Notre Dame).

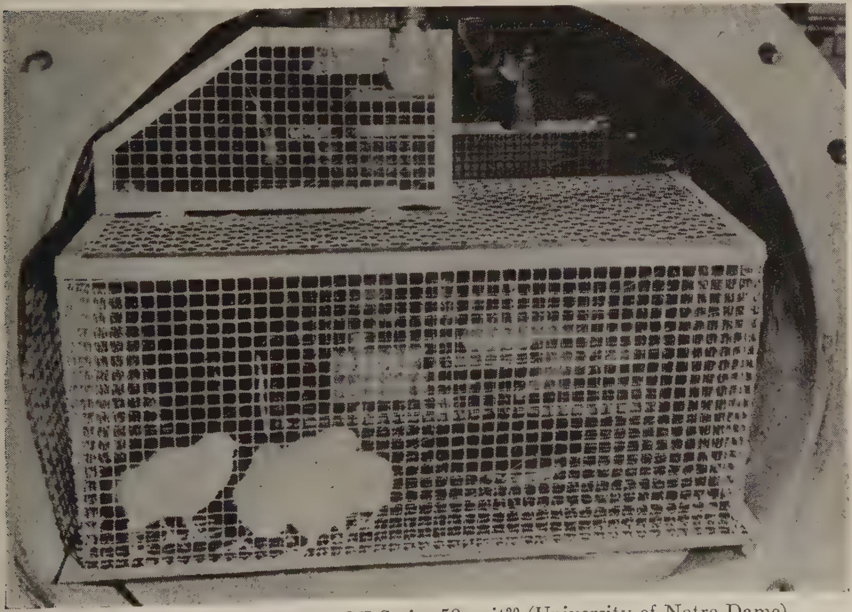

FIGURE 6. Interior of the RGF Series 50 unit[20] (University of Notre Dame).

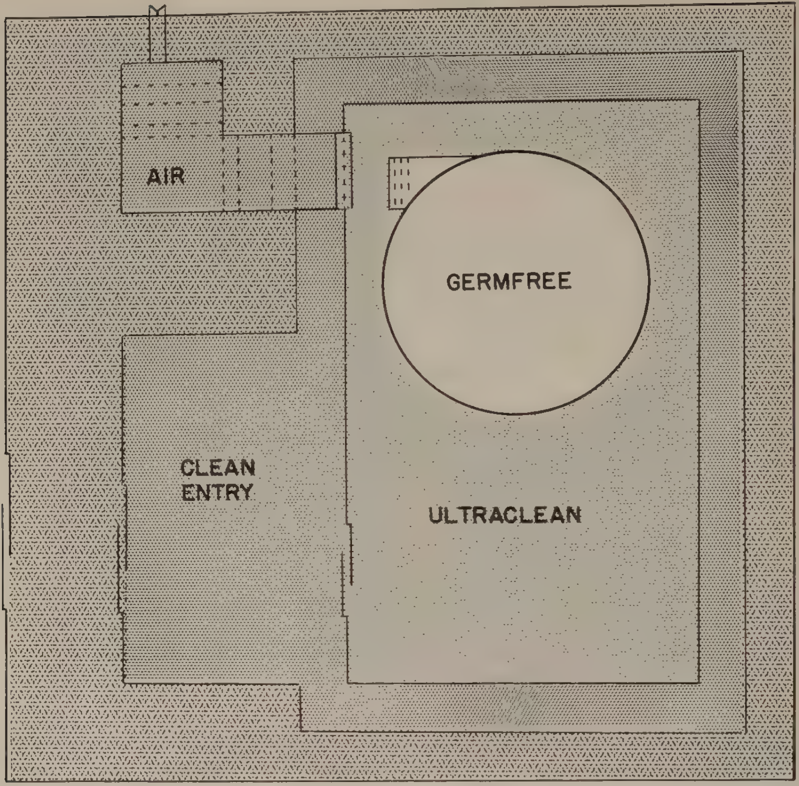

Figure 7. Autosterilizing room for rearing germfree animals (University of Missouri).

Tables 1 and 2 give the composition of the diets used in this work. There are both practical and synthetic (syntype) diets. The diet for the vitamin deficiencies was standardized so that one basic diet (L-245) could be used, the only modification being the omission of the vitamin under study. Practicable diets were made with various purposes in mind.

The sterilization of the diet constitutes a major problem. This can be done by a variety of methods, depending upon what is fed. Early in our work we tried to feed liquid diets to chicks. While such diets appear to be utilized by mammals very well (particularly milk diets for suckling mammals), chicks find the diet much like a good glue and become as helpless as Br'er Rabbit in his encounter with the tar baby. However, small amounts of liquid are sometimes used in adding vitamin supplements to a large quantity of solid food. Sometimes the liquid was sterilized by heat; sometimes it was filtered. Intermittent heat sterilization was used on some of the grain diets used in experiments at the University of Wisconsin, but it was found to be a very laborious process and certainly seemed to be as deleterious to the diet as steam autoclaving it in thin layers. Consequently autoclaving was used for all of the diets reported

TABLE 1

COMPOSITION OF DIETS

(Grams)

Constituents	L-137	56227[§]	L-245	L-171[¶]
Salts L-II[*]	50	50	50	70
Fiber, Alphacel	—	—	—	—
Fiber, Celluflour	—	—	60	—
Starch, corn	450	549	510	—
β-Lactose	40	0.9	—	—
Oil, corn	60	43	50	54
Casein, Labco	250	250	250	229
Gelatin	100	100	—	—
Glycine	—	—	5	—
L-Cystine	2	3	4	—
L-Arginine HCl	—	—	3	—
Vitamin A (I.U.)	8,000	50,000	6,000	16,190
Thiamin HCl	0.06	0.016	0.05	0.021
Riboflavin	0.03	0.016	0.02	0.017
Nicotinamide	0.05	0.05	0.10	0.062
Choline Cl	2.0	2.0	2.0	3.2
Ca pantothenate	0.2	0.044	0.10	0.13
Pyridoxine Cl	0.02	0.012	0.02	0.010
Biotin	0.0004	0.0004	0.0005	0.0004
Folic acid	0.01	0.005	0.02	0.014
Inositol	1.0	1.0	1.0	1.5
Vitamin B_{12}[†]	—	—	0.00002	—
Yeast extract (powder)	20	—	—	—
Liver, whole powder	20	—	—	—
Ascorbic acid (powder)	2.0	—	1.0	—
Vitamin D_3 (I.U.)	1,000	5,000	1,000	1,060
Vitamin E	0.5[‡]	2	0.3	0.53
Vitamin K	0.1	0.05	0.01	0.1
p-Aminobenzoic acid	—	0.1	—	—

[*] The composition of salts L-II in grams per kilogram of diet is: $CaCO_3$, 15.0; $CaHPO_4$, 2.75; K_2HPO_4, 11.3; Na_2HPO_4, 10.0; NaCl, 2.5; KI, .0375; $MgSO_4 \cdot 7H_2O$, 3.5; $MnSO_4 \cdot 4H_2O$, 0.625; $Fe(C_6H_5O_7)_2$, 3.75; $CuSO_4 \cdot 5H_2O$, 0.19; $CoCl_2 \cdot 6H_2O$, 0.025; $ZnSO_4 \cdot 7H_2O$, 0.05; $Na_2B_4O_7 \cdot 10H_2O$, 0.025; and $AlK(SO_4)_2 \cdot 10H_2O$, 0.0375.

[†] Supplied by Merck & Co., Inc., Philadelphia, Pa., as a concentrate.

[‡] Alpha-tocopherol was used for these diets.

[§] This diet, obtained from Parke, Davis & Company, Detroit, Mich., used lard in place of corn oil and the Jones and Foster salt mix with 0.1 per cent $MnSO_4 \cdot 4H_2O$.

[¶] Calculated composition. The total protein of this diet is given on the line of casein.

herein. Usually the material is spread in a one-half-inch layer on cloth and is sterilized at 17 psi for 20 min. As one would expect, the diet is changed by autoclaving; TABLE 3 shows the change in nutrients. The protein remains about the same and the lipid appears to decrease somewhat, possibly because it is absorbed by the cloth surrounding the diet. The loss in B vitamins is one of the most important factors to note. This loss was made up simply by adding an excess of each of the given vitamins. When we expect 90 or 95 per cent of the thiamin to be lost we make this up by adding 10 or 20 times more thiamin that is needed. Apparently neither the excess vitamins nor their degradation products, which must be present in the autoclaved diet, have any materially adverse effects on the chickens. As mentioned above, filtration is sometimes

TABLE 2
COMPOSITION OF PRACTICAL DIET
(Grams)

Constituents	L-189	171
Corn, cracked	490	—
Oil, corn	—	20
Soya meal(s)	232	—
Alfalfa leaf meal	28	50
Whole wheat flour	—	560
Wheat mids	93	—
Buttermilk powder	—	150
Fish meal	23	—
Yeast, brewers	—	100
Meat scrap	23	50
Liver, fraction B	—	20
Salt, iodized	4.7	—
$CaCO_3$	16.7	—
Ca_2HPO_4	14.9	—
$MnSO_4 \cdot H_2O$	0.24	—
Casein	70	—
Salts L-II	—	30
Vitamin A	9200	4000
Vitamin D	2300	500
Vitamin E	—	0.025
Vitamin K	—	0.005
Thiamin	—	0.015
Pantothenic acid	—	0.10
Folic acid	—	0.005
Biotin	—	0.0002

TABLE 3
NUTRIENT LOSS IN SYNTYPE DIET (L-245)

	Fat	Protein	Vitamin A	Vitamin E	Vitamin C	Thiamin	Riboflavin	Pantothenate	Niacinamide	Choline	Pyridoxine	Biotin	Folate	Vitamin B_{12}
(1)	0	10	—	—	—	45	34	11	2	—	—	0	40	38
(2)	11	11	33	50	20	92	54	25	17	3	20	30	62	50

(1) Percentage lost by mixing procedure.
(2) Percentage lost during mixing, steam sterilization (17 psi for 15 min.), and storage for one week at room temperature.

used to sterilize material; it was used to sterilize the fresh liver extract fed to the chicks reported here. Filtration has also been used for parts of diets when liquid diets were fed to germfree animals. Gamma-ray sterilization of syntype diets is quite feasible, as has been shown by Luckey *et al.*,[21] but it has not been used extensively for complete diets for germfree chicks. However, gamma rays were used to sterilize the antibiotics fed to germfree chicks and turkeys. Ethylene oxide-sterilized diets have been fed to chicks that were not sterile. Apparently the diet is not harmed materially, because the general appearance and growth of the chicks was satisfactory. A seventh method of sterilizing food is, of course, a combination of one or more of the above. Two examples are the filtered fresh liver extract and X-ray-treated antibiotics added to steam-sterilized diet and mixed in the cage. Combinations of a filtered solution of casein and a separate solution for carbohydrate and salt have been used for sterile rats. Perhaps another method for procuring sterile food should be mentioned: that is, to grow sterile food to be fed to sterile animals. To my knowledge, the only time this has been used was when sterile chickens were fed to sterile rats in an effort to obtain reproduction with lactation.

It should be noted that sterile diets are not necessarily free from bacteria. Syntype diets are made from normal, commercially obtainable ingredients such as vitamins, mineral, salts, corn oil, carbohydrate, and casein. Small quantities of the dead bacteria appear in the diet as fed to the chicken. Some of these bacteria come from the hands of the people who mix the food.[22] Many of these dead organisms go through the gastrointestinal tract and are excreted in fecal material. Some parts of them must be absorbed, for Wagner reports[22] that antibody response is obtained after a long period of maintaining germfree chicks on these diets. It should not be difficult to obtain an antigenetic diet, but thus far the opportunity to use such a diet effectively has not presented itself.

The methods for testing sterility of the cages, of the air, of the equipment, of the food, and of the animals will not be discussed here, as Wagner deals with them adequately elsewhere in these pages. One difficult and tedious task throughout each and every given germfree experiment that should be discussed is a satisfactory system for testing for viruses. Ignoring for the moment the question of whether any organism can be virus-free, is it not possible to test for one or two of the chicken viruses that are most predominant in order to determine that experiments with chickens are virus-free to some degree? I suspect that the virus-free state will be of equal importance to germfree state in the future.

The analytical methods used are those reported previously.[23] It may be reiterated that they were standardized to allow the maximum amount of information from the small samples available.

Results

Nutrition, growth, and general appearance. The first experiments with chicks kept on practical diets were rather disappointing, as illustrated by FIGURE 8. The germfree chicks grew very little, while control chicks on the nonsterilized diet grew satisfactorily. To our surprise, no increased growth rate was obtained when an excess of vitamins was added to diet L-171. Acceptable growth was obtained when increased protein was added with the vitamin supplementation. Consequently, it is apparent that protein denaturation is a major factor in the sterilization of the practical diet. Similar results have been obtained with rats. The other major factor to be considered in the sterilization of practical diets is the destruction of thiamin. While we often think in terms of 90 per cent of the thiamin being destroyed, it is apparent from some experiments that 95 per cent or possibly more is destroyed when a practical diet is used. Therefore any time germfree chicks are found to be constantly eating without growing, one should look into the thiamin content of the diet. Thiamin deficiency in these chicks does not necessarily lead to anorexia as is classically expected. The picture is not at all the same with syntype diets.

Germfree chicks grow quite satisfactorily when they are kept on a syntype diet, as shown by the Bantam chicks in FIGURE 9. These chickens grew quite well and maintained life when maintained on such diet (L-137). Additions of natural products as sources of unidentified vitamins appear to give no enchanced rate of growth (TABLE 4) in Leghorn chicks.[23] Egg production and reproduction was also obtained[24] when 1 per cent more minerals was added to the diet of White Wyandotte chickens. The only second generation germfree chick obtained was apparently not healthy and died shortly after hatching. Autopsy revealed white deposits throughout much of the viscera grossly similar to those reported in vitamin A deficiency.

Anal pasting (FIGURE 10) was seen sporadically in both germfree and conventional chicks during one period of several months' duration. Anal pasting is the complete blockage of the anus by an accumulation of excreta sticking to the perianal skin. By gross inspection, the feces appeared to contain more mucin than usual and possibly less ureates. Death occurs within 2 weeks if the condition is not corrected. It was seen in both White Leghorn and White Wyandotte Bantam chicks, on both syntype and practical diets. Thiamin deficiency accentuated the syndrome. Lowering the protein, eliminating the

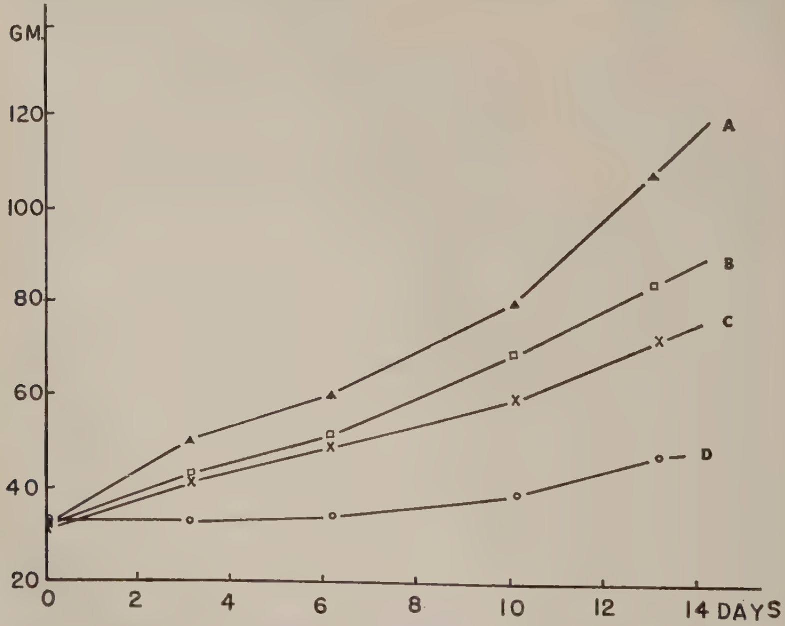

FIGURE 8. White Leghorn chicks fed crude rations: *A*, control chicks on nonsterilized diet; *B*, germfree chicks given added proteins and vitamins; *C*, control chicks; *D*, germfree chicks with and without vitamin supplement.

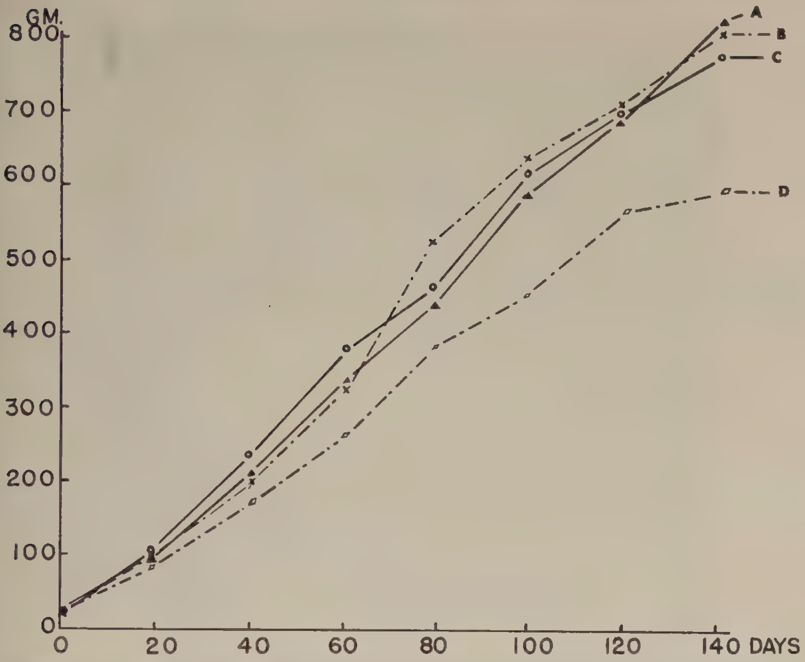

FIGURE 9. Growth of Bantam chicks on syntype (L-137) diet: *A*, control (1 female); *B* ermfree (3 males); *C*, control (3 males); *D*, germfree (1 female).

TABLE 4
EFFECT OF NATURAL PRODUCTS ON GROWTH OF GERMFREE CHICKS*

	Diet	N	M	Range
(1)	56227	2	287	(282 to 292)
(2)	56227 + 2 per cent yeast extract + 2 per cent liver powder	1	298	
	+ 2 per cent fermentation residue	5	248	(208 to 298)
(3)	as 2 + fresh liver extract	5	264	(213 to 295)

* Weight of 4-week-old White Leghorn chicks.

ctose, and adding fiber to the diets appeared to prevent this condition. Diet -189 was formulated with these conditions in mind.

Composition. The chemical composition of germfree chicks on syntype diet -245 was examined (FIGURES 11 to 25). Data obtained from conventional hicks on the same sterilized diets are compared to those from germfree chicks. his is done from the consideration that the presence of microorganisms in onventional chicks is essentially the result of uncontrolled variables. The ase line in the figures represents the germfree bird taken as the control. The ean values for the germfree birds are given below with the numbers of animals each category and the significance of the differences noted by the bar. The

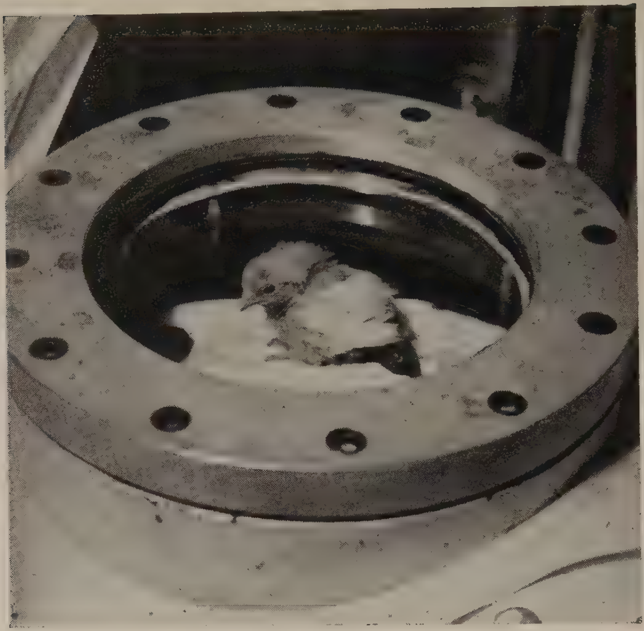

FIGURE 10. Germfree chick suffering from anal pasting.

solid bar indicates the differences noted in conventional chicks (with microflora). The small discontinuous bars indicate the coefficient of variance ($\sigma/M \times 100$); that on the left is from germfree data and that on the right is from conventional chicks. These data are on a fresh-weight basis.

Conventional White Leghorn chicks are found to have the same amount of dry weight (FIGURE 11) as germfree chicks in tissues from kidney, liver, muscle, brain, lung, small intestinal wall, and rectal contents. The bile and cecal contents contain less moisture in conventional birds than was found in germfree birds. The ash content (FIGURE 12) of liver, brain, lung tissues, and the rectal contents of conventional birds is lower than that of germfree birds. No differences were found in the ash content of kidney, muscle, bile, small intestinal wall, cecal contents, bone, or total chick.

No differences were found in the total nitrogen (crude protein) content for kidney, liver, muscle, brain, bile, cecal contents, lung, or bone (FIGURE 13). The values obtained for cecal contents, of course, would be more nearly the same on a dry-weight basis. The finding that the small intestine of conventional birds has less nitrogen than that of germfree birds is of special interest, since Gordon[25] has found the intestinal walls of germfree birds to be lighter than that of conventional birds. Presumably the additional material found in conventional birds is lower in protein than the "unstimulated" intestinal wall of germfree birds.

A similar statement might be made for the fat content of the intestinal wall, FIGURE 14 (since the water and ash contents were not different, it would ap-

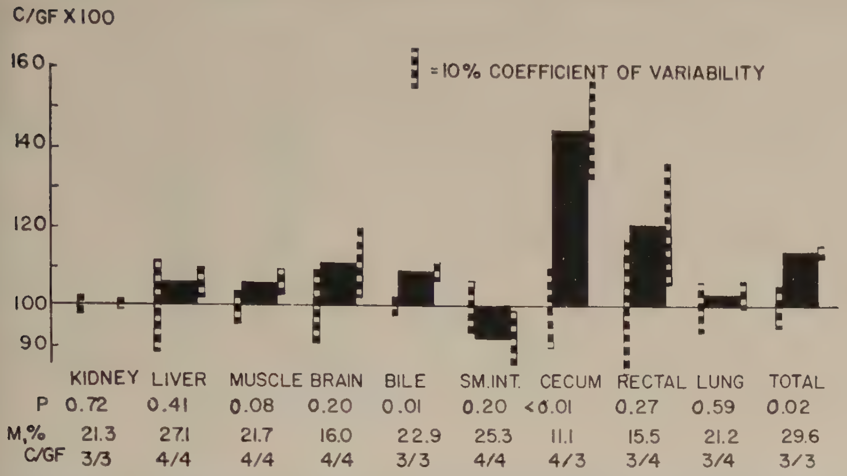

FIGURE 11. Dry weight of tissues of 10-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,%, mean percentage value.

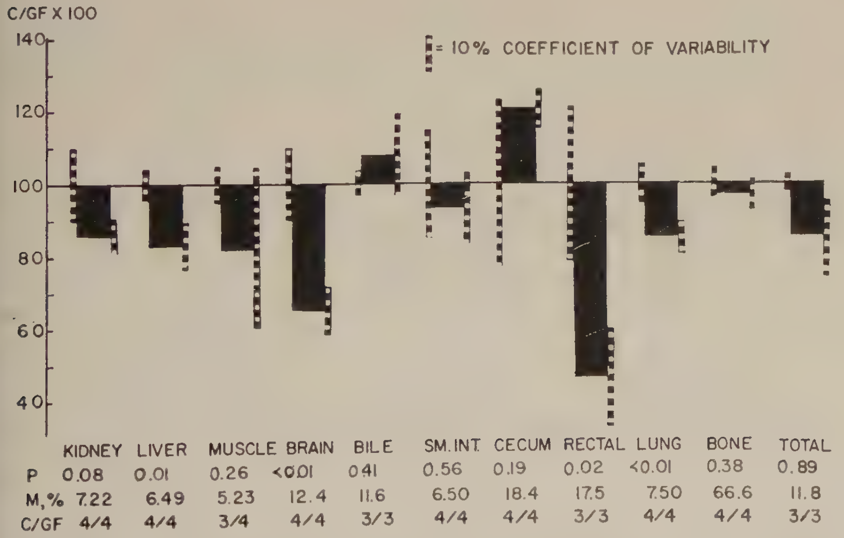

FIGURE 12. Dry weight of ash in tissues of 10-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,%, mean percentage value.

pear to be important to examine the carbohydrate content of this tissue). No differences were seen in the fat content of kidney, liver, muscle, brain, or bone. Lung tissue of conventional birds contains only three quarters as much fat as is found in the lungs of germfree birds.

Although the vitamin C content of "critical" tissues (that is, critical in the

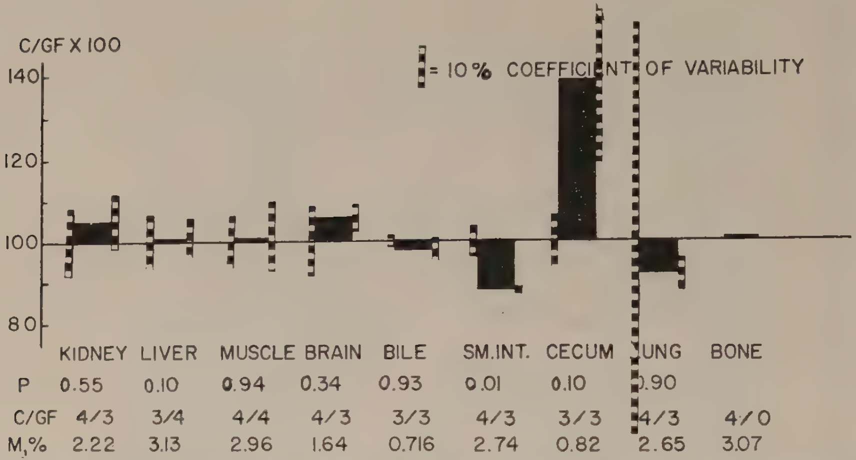

FIGURE 13. Nitrogen content of tissues of 10-week-old White Leghorn chicks. Symbols: C/GF, ratio of germfree to conventional birds; P, probability ratio; M,%, mean percentage value.

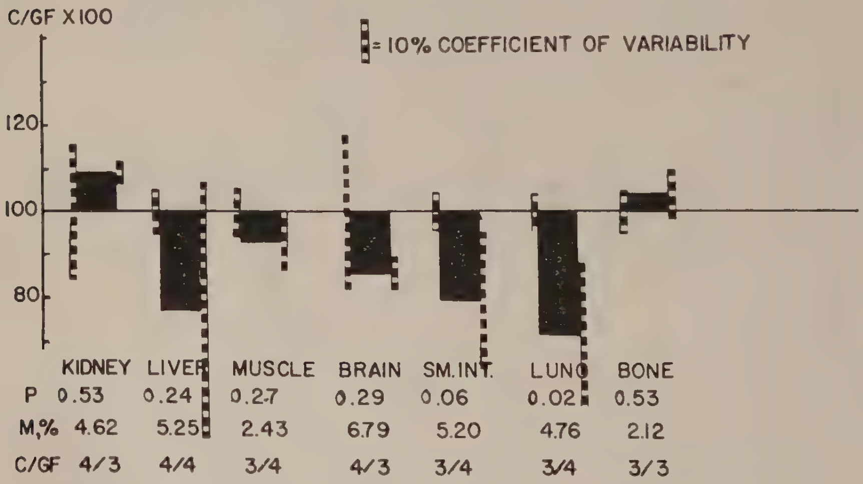

FIGURE 14. Fat content of tissues of 10-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability; M,%, mean percentage value.

metabolism of vitamin C) such as adrenal gland and brain show no differences (FIGURE 15), the ascorbic acid content of liver and muscle was found to be greatly increased in birds harboring microorganisms.

The riboflavin content of the bile of conventional birds (FIGURE 16) was only one quarter as much as is present in germfree birds. No differences can be seen in the riboflavin content of liver, muscle, brain, cecal contents, or bone.

Although 2.62 γ of niacin per gram of tissue was found in the blood of germfree chicks (FIGURE 17), none was detected in the blood for conventional chicks. The niacin content of cecal contents was threefold higher in conventional chicks

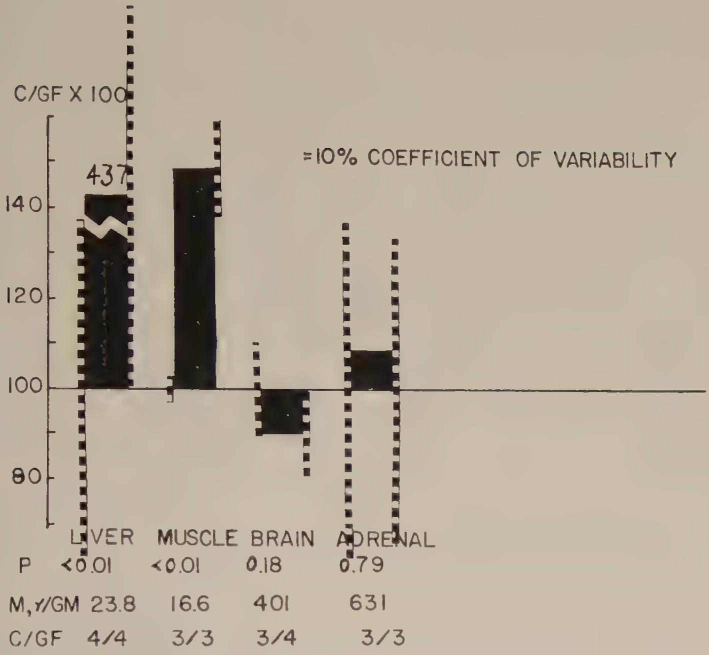

FIGURE 15. Vitamin C content of tissues of 4- to 7-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio, M,γ/gm., mean in terms of micrograms of vitamins per gram of tissue.

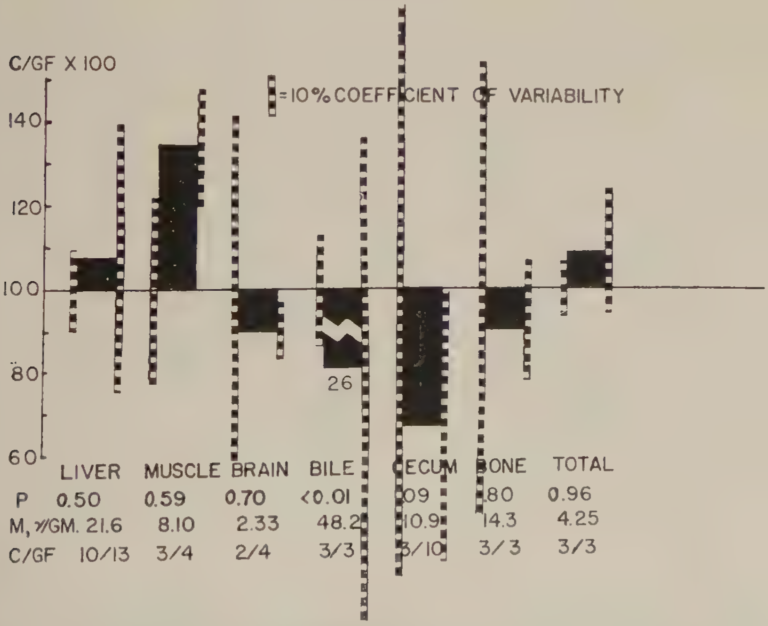

FIGURE 16. Riboflavin content of tissues of 4- to 7-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,γ/gm., mean in terms of micrograms of riboflavin per gram of tissue.

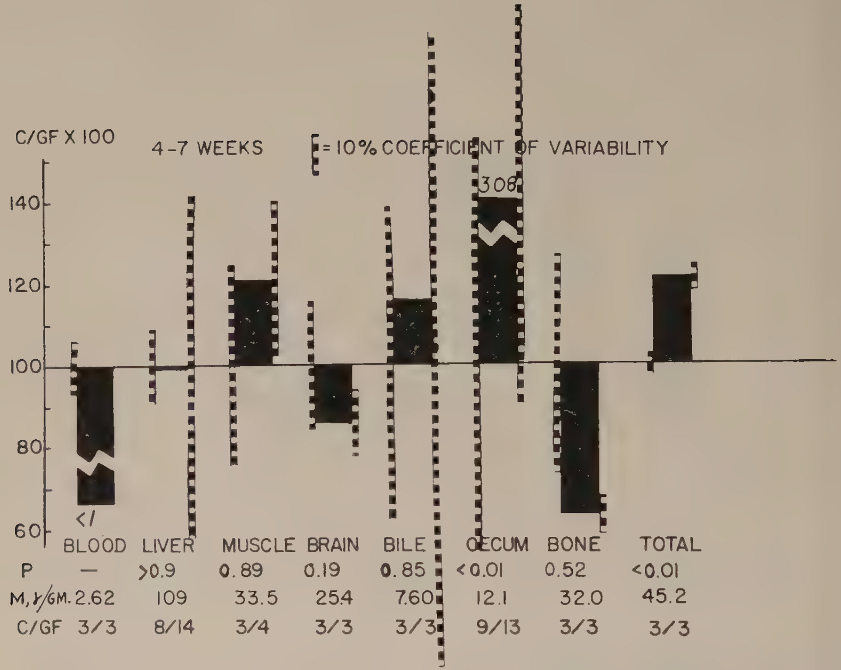

FIGURE 17. Niacin content of tissues of 4- to 7-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,γ/gm., mean in terms of micrograms of niacin per gram of tissue.

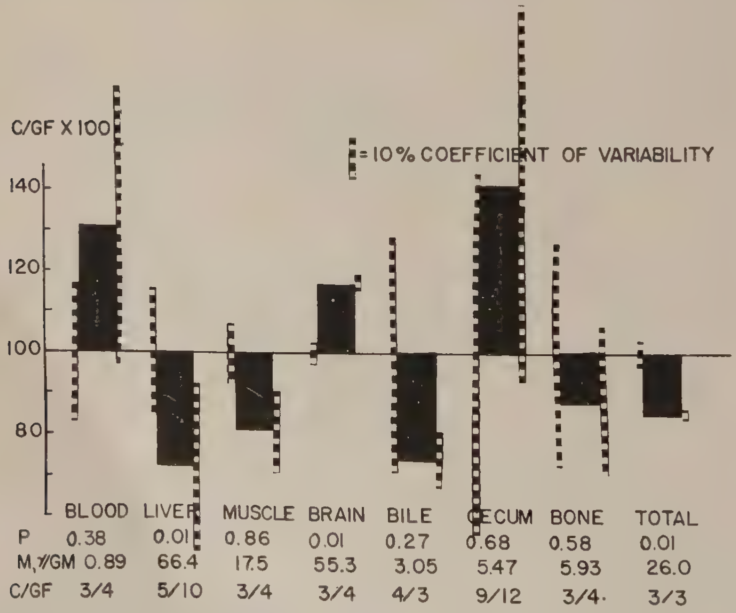

FIGURE 18. Pantothenate content of tissues of 4- to 7-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, ratio of probability; M,γ/gm., mean in terms of micrograms of pantothenate per gram of tissue.

an in germfree chicks. No differences were found in the niacin content of ver, muscle, brain, bile, or bone.

The pantothenic acid content (FIGURE 18) of blood, muscle, bile, cecal con-ents, and bone were similar in germfree and conventional birds. This vitamin ppeared to be somewhat high in brain and low in liver tissue in conventional irds. The total carcass of conventional birds was high in niacin and low in antothenate.

Biotin was found to be comparable (FIGURE 19) in blood, liver, muscle, brain, ecal contents, and bone of germfree and conventional birds. The bile of onventional birds contained less than one third as much biotin as was found the bile of germfree chicks.

The liver tissue of conventional chicks contained less folic acid (FIGURE 20) nd vitamin B_{12} (FIGURE 21) than did "normal" liver tissue. No changes ere noted in the folic acid content of blood, muscle, brain, bile, cecum, or bone, or were differences seen in vitamin B_{12} content of blood, muscle, brain, or one. Further work is needed on bile and cecal contents.

No dramatic differences were seen between the thiamin, pyridoxine, or hosphorus content of the livers of germfree and conventional chicks (FIGURE 2), nor was there significant difference seen in the phosphorus content of muscle ssue in the two groups.

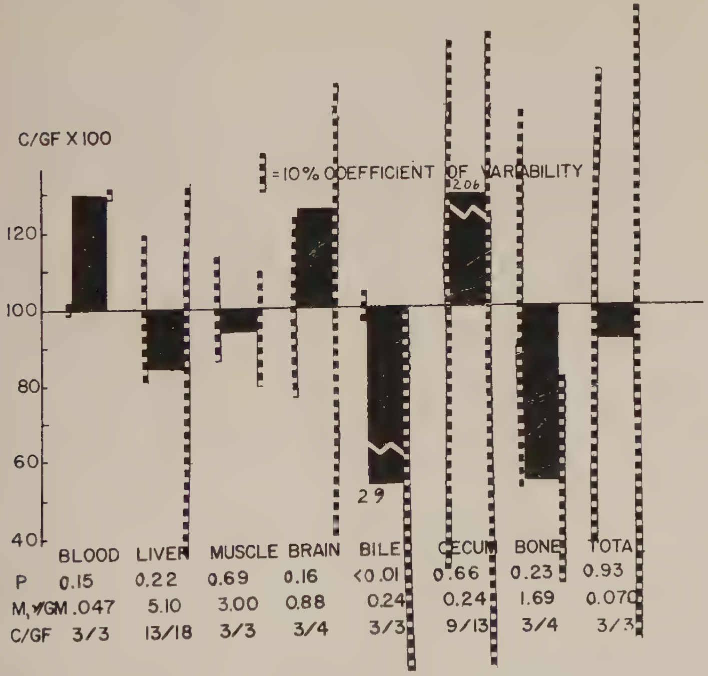

FIGURE 19. Biotin content of tissues of 4- to 7-week-old White Leghorn chicks. Sym-ols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,γ/gm., mean in rms of micrograms of biotin per gram of tissue.

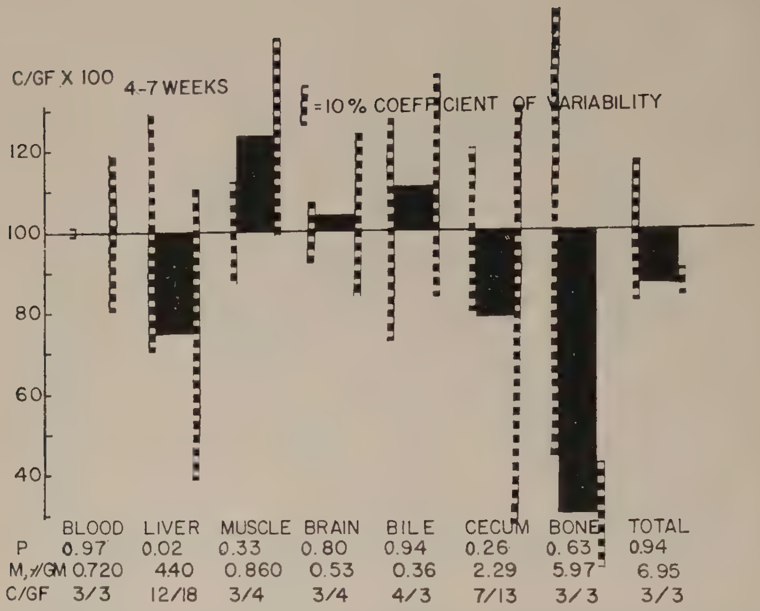

FIGURE 20. Folic acid content of tissues of 4- to 7-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,γ/gm., mean in terms of micrograms of folic acid per gram of tissue.

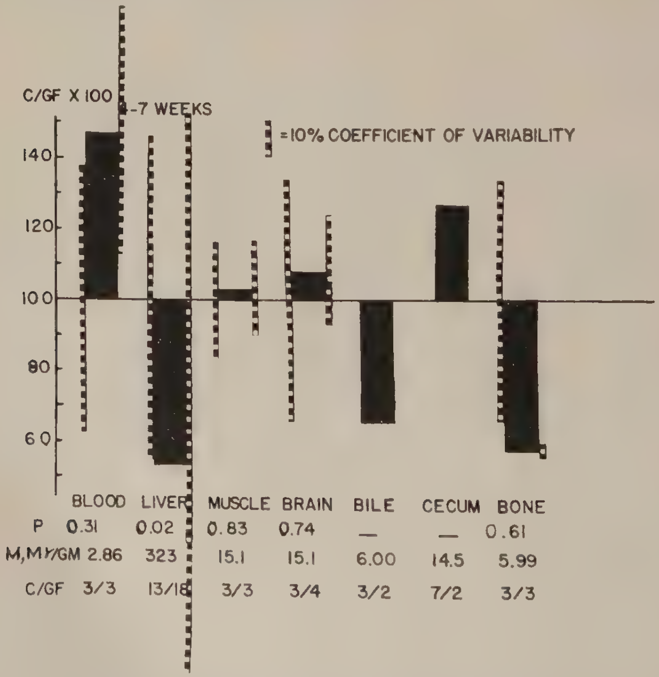

FIGURE 21. Vitamin B_{12} content of tissues of 4- to 7-week-old White Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M, mγ/gm, mean in terms of millimicrograms of vitamin B_{12} per gram of tissue.

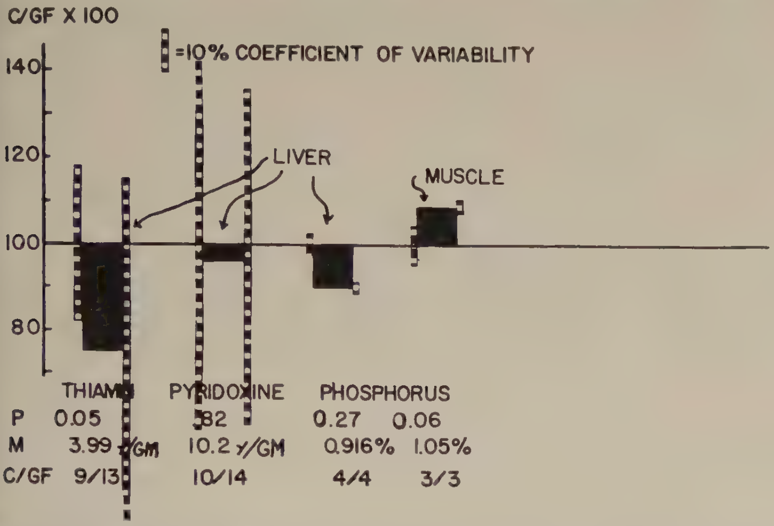

FIGURE 22. Thiamin, pyridoxine, and phosphorus content of tissues of 4- to 7-week-old 'hite Leghorn chicks. Symbols: C/GF, ratio of conventional to germfree chicks; P, prob- ›ility ratio; M, γ/gm., mean in terms of micrograms per gram of tissue.

TABLE 5

VITAMIN ANALYSES (LEGHORN CHICKS)

	Riboflavin	Niacin	Pantothenate	Biotin	Folic acid	Vit. B_{12}
			Diet			
	10.3	92	34	0.39	2.8	0.033
			Cecal contents			
onventional.......	46.6	254	115	8.3	11.7	0.096
ermfree..........	37.7	105	264	5.9	10.5	0.109
			Apparent bioincrassation			
ermfree..........	3.7	1.1	7.8	15.1	3.8	3.3

The data give the average γ vitamin/gm. dry material from 8 to 31 chicks.

When the concentration of vitamins in the cecal contents is compared with iat of the diet on a dry-weight basis, the material in the ceca is found to have ›nsiderably greater concentrations of vitamin than the diet; this was pre- ously considered to be a part of the phenomenon of intestinal synthesis. ata from germfree chicks (TABLE 5) indicates the same phenomenon occurs ithout the action of any intestinal microorganism. A similar effect is seen hen dead bacteria[20] or mineral content of the diet and the feces are compared. is suggested that this apparent accumulation occurs because a greater propor- ›n of carbohydrate, fat, and protein are absorbed than are molecules that are not absorbed well, such as vitamins. The net effect is to concentrate the vitamins in the gastrointestinal tract by taking other solid material (carbohydrate, fat, and protein) away from the vitamin. Therefore this phenomenon has been termed "bioincrassation."

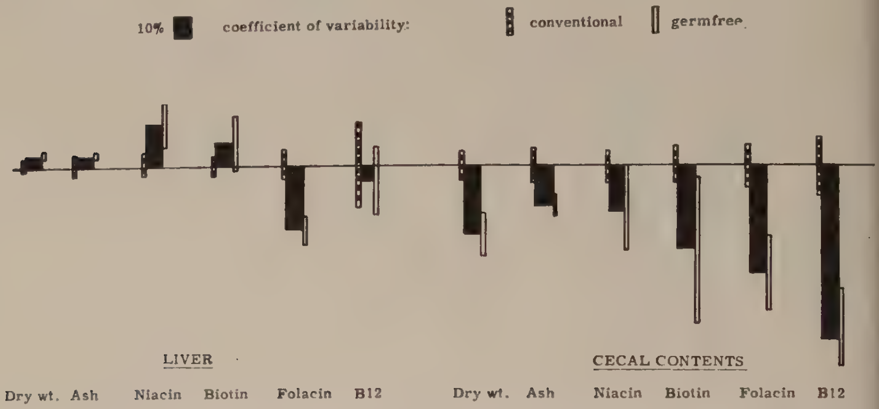

FIGURE 23. Biochemical comparison of germfree to conventional chicks on diet L-289.

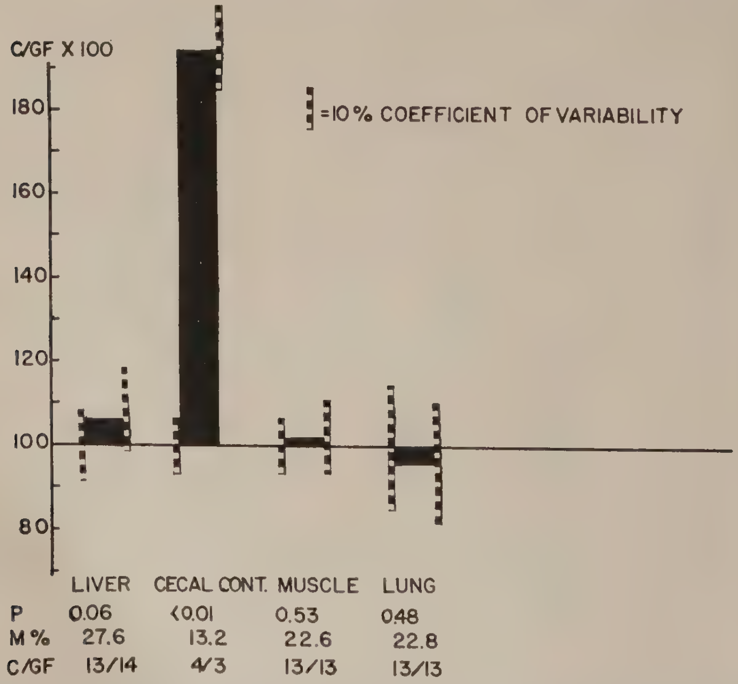

FIGURE 24. Dry weight of tissues of 4- to 8-week-old White Wyandotte Bantam chicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio, M,%, mean percentage value.

A less-detailed study of biochemical constitution of chicks kept on a practical iet (L-189) is summarized in FIGURE 23 (note that the germfree bird is compared) conventional chicks in the bar graph; previously the conventional chicks were ompared to germfree chicks). No changes are seen in the liver content of ry matter, ash, biotin, or vitamin B_{12}. The quantity of liver niacin may be omewhat low, and the concentration of folic acid is high in conventional irds as compared with germfree birds. Several differences seem apparent etween the cecal contents of these groups of birds when the values are conidered on a wet-weight basis. Since the dry weight of the cecal contents is igher in conventional birds than in germfree birds, some of the apparent diferences would disappear if the values were considered on a dry-weight basis. 'he ash, folic acid, and vitamin B_{12} in cecal contents of germfree birds is efinitely lower than that of conventional chicks when both are kept on this ractical diet.

Analysis of White Wyandotte Bantam chicks kept on the syntype diet L-137 gain showed the cecal contents of conventional birds contained more dry eight than do the cecal contents of germfree birds (FIGURE 24). No differences ere seen in the moisture content of liver, muscle, or lungs. Further analysis f the livers of these birds (FIGURE 25) indicated no differences between germree and conventional chicks in ash, nitrogen, phosphorus, fat, thiamin, riboavin, pyridoxine, biotin, or folic acid. These data generally agree with those aken from White Leghorn chicks, except that ash, pyridoxine, and folic acid ere lower in conventional than in germfree White Leghorn chicks.

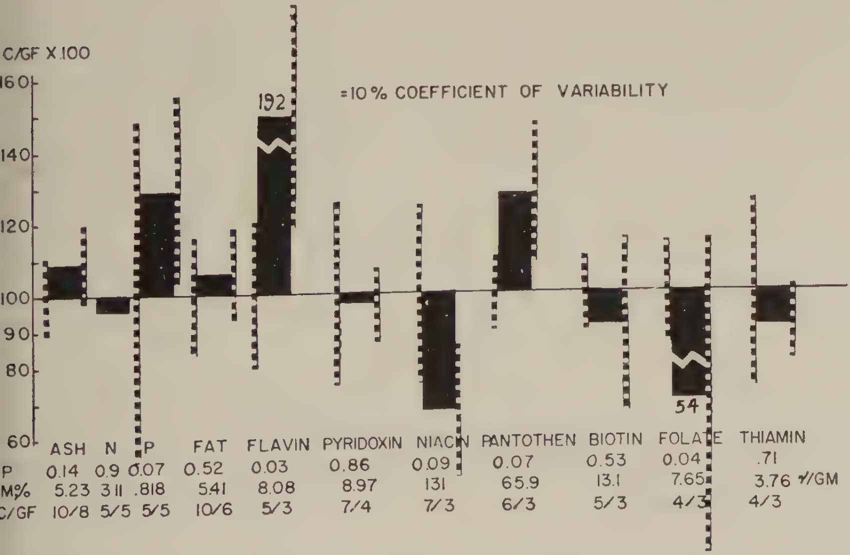

FIGURE 25. Biochemistry (liver analysis) of 7- to 9-week-old White Wyandotte Bantam hicks. Symbols: C/GF, ratio of conventional to germfree birds; P, probability ratio; M,%, nean percentage value; γ/gm., mean in terms of micrograms per gram of tissue.

Vitamin Requirements

The qualitative requirement for vitamins by germfree chicks is illustrated in FIGURE 26. Germfree chicks obviously require thiamin, riboflavin, niacin, biotin, and folic acid. Details of each deficiency examined are reported elsewhere.[26] Vitamin analyses of deficient tissues were performed on deficient chicks usually about 3 weeks old.

Vitamin A deficiency. Germfree chicks fed the syntype diet with vitamin A omitted grew at a somewhat slower rate than chicks on the complete diet (L-245), as shown in FIGURE 26. At two weeks they exhibited leg weakness, and at 3 weeks the ataxia was pronounced. The deficient germfree birds grew somewhat faster than did polybiotic (meaning multicontaminated or conventional) chicks on the deficient diet. Three of the 8 germfree chicks died during the fourth week of the experiment.

Thiamin deficiency. Results of maintaining 18 germfree birds on a thiamin-deficient diet, reported in detail elsewhere,[26] are summarized in FIGURE 26. No differences are noted between germfree and conventional chicks on the thiamin-deficient diet. All chicks in both groups exhibited anorexia, lack of growth, and ataxia, and they died in 9 to 12 days. Many had cervical tics, and some were found with opisthotonos. Injection of thiamin into paralyzed chicks of both groups brought a marked, but temporary, improvement in apparent disability.

Livers of both germfree and conventional thiamin-deficient chicks at 11±

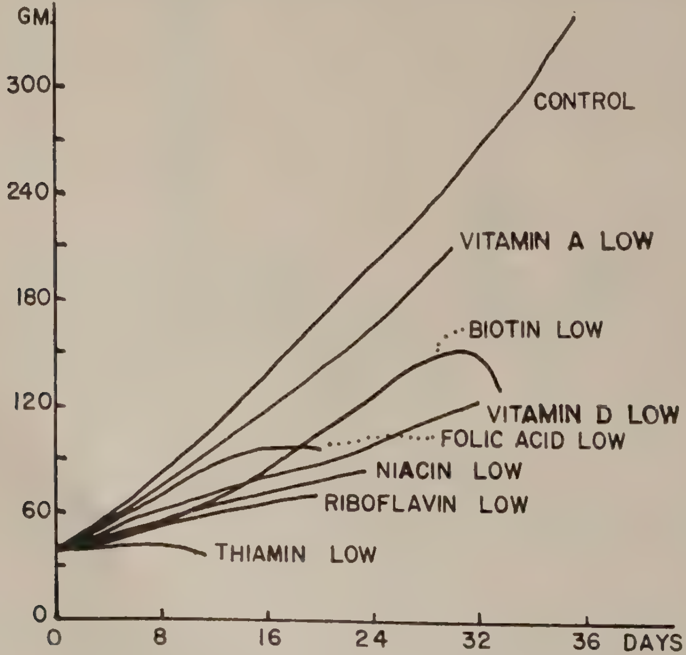

FIGURE 26. Growth of germfree White Leghorn chicks.

days (usually moribund) were much lower in thiamin than in 4-week-old chicks on the complete diet (TABLES 6 and 7). Riboflavin, niacin, pantothenic acid, biotin, folic acid, and vitamin B_{12} concentrations were similar in the livers of deficient and normal chicks, whether or not they were germfree. Only in the case of riboflavin did the germfree birds' livers have a lower vitamin content than those of the conventional chicks when both were kept on thiamin-deficient diets. There are too few data from cecal content analysis to allow any comparisons.

In one experiment in which too much thiamin was evidently destroyed during sterilization, both germfree and conventional chicks failed to grow properly. Oral supplementation of thiamin at 10 to 20 days allowed acceptable performance in the birds as shown by the growth curve (FIGURE 27). It became necessary to supplement the diets of the conventional birds again at 50 days to prevent severe deficiency, and the germfree birds were given a third supply at 30 days. This supply lasted the conventional birds for about 3 weeks, after which time they rapidly lost weight and died. The germfree birds became contaminated with a *Staphylococcus* species about 3 weeks after the third supplement was given to them, and they showed no further ill effects from the shortage of thiamin. It is not known whether this result may be attributed to increased cell permeability, intestinal synthesis, or increased utilization of the small amount of residual thiamin in the diet.

Riboflavin deficiency. A severe and acute riboflavin deficiency was obtained in 11 germfree chicks. These birds grew at about the same rate as polybiotic (conventional) chicks on the same diet, but muscular paralysis and mortality were more severe. Analysis of the livers of riboflavin-deficient chicks (TABLES 6 and 7) at 3 weeks showed no decrease in thiamin, pantothenic acid, biotin, or folic acid in either the germfree or the polybiotic state, nor were differences noted between germfree and conventional chicks in the vitamin content of the liver except in the case of niacin. As expected, the riboflavin content of the livers was lower than in chicks on the complete diet. In the livers of germfree birds with riboflavin deficiency niacin was found to be low. No good explanation for the effect of riboflavin on niacin metabolism is offered.

The biotin in cecal contents of riboflavin-deficient germfree birds appeared to be much higher than in either deficient conventional birds or germfree birds on the complete diet.

Niacin deficiency. This condition appeared to be more severe in the 5 germfree birds used than in conventional birds. Growth and feather formation were very retarded in the germfree birds.

Surprisingly, the niacin content of the liver of niacin-deficient birds was not low (TABLES 6 and 7). The niacin and riboflavin in the liver of the germfree birds was significantly higher than in niacin-fed germfree birds. Thiamin difference almost showed significance. No differences were seen in the liver content of pantothenic acid, biotin, or folic acid.

Cecal content analysis indicated that riboflavin was lower in germfree birds than in conventional birds. This adds to the interrelationships noted between niacin and riboflavin. No significant change was seen in the quantity of niacin in the cecum of birds on diets high or lacking in niacin.

TABLE 6
ANALYSIS OF THE LIVERS OF GERMFREE AND CONVENTIONAL CHICKS

Analysis	Complete diet		Diet deficient in									
			Thiamin		Riboflavin		Niacin		Folic acid		Vitamin K	
	GF	Conv.	GF	Conv.	GF	Conv.	GF	Conv.	GF	Conv.	GF	Conv.
Thiamin N	9	13	3	9	8	5	7	10	9	10	8	6
M	3.99	3.00	0.824	0.647	3.27	3.86	4.94	4.26	2.49	2.42	4.52	4.84
σ	0.723	1.27	0.830	0.388	1.09	0.710	0.894	0.734	0.564	0.816	0.413	4.50
P_1			<0.01	<.001	0.148	0.23	0.041	0.013	<0.001	0.27	<0.001	0.24
P_2	0.048		0.686		0.36		0.125		0.56		0.86	
Flavin N	10	13	3	8	8	5	7	10	9	11	7	6
M	21.6	23.3	17.5	25.0	7.1	12.5	34.7	22.7	34.9	19.2	16.1	10.9
σ	2.06	7.25	5.45	1.97	4.98	2.98	17.7	10.1	7.37	5.79	4.75	3.91
P_1			0.067	0.155	0.001	<0.001	<0.001	0.53	<0.001	0.49	<0.001	<0.001
P_2	0.50		0.016		0.076		0.117		0.002		0.069	
Niacin N	8	14	3	6	8	5	7	10	9	11	8	6
M	109	108	69.3	104	86	123	145	110	104	87	130	141
σ	9.9	45.1	16.1	140	21.3	32.9	3.94	22.9	30.9	40.8	16.0	15.9
P_1			0.003	0.85	<0.001	0.53	<0.001	0.69		0.69	0.023	0.110
P_2	0.94		0.43		0.016		<0.001		0.55		0.001	
Ca pantothenate N	5	10	3	7	8	5	7	10	9	11	8	6
M	66.4	48.0	47.6	45.0	57.7	47.3	54	51	17.6	15.3	19.6	17.9
σ	10.4	9.9	18.9	12.9	27.9	13.3	8.24	9.70	2.48	6.77	4.30	12.2
P_1			0.153	0.63	0.53	0.90	0.18	0.40	<0.001	<0.001	<0.001	<0.001
P_2	0.001		0.82		0.63		0.12		0.38		0.9+	

Biotin N	13	13	3	6	7	5	7	10	9	11	8	6
M	5.14	4.31	4.47	4.70	4.3	4.0	5.1	6.5	4.15	3.49	4.7	3.7
σ	1.0	2.1	1.27	1.68	1.18	0.574	2.55	2.55	1.47	1.79	1.63	0.843
P_1			0.43	0.83	0.94	0.66	0.94	0.037	0.078	0.9+	0.47	0.52
P_2	0.215		0.87		0.64		0.27		0.41		0.88	
Folic acid N	12	18	3	7	8	5	7	10	12	14	8	6
M	4.41	3.27	3.70	2.96	5.2	2.8	2.9	2.3	3.60	2.31	5.0	3.4
σ	1.26	1.15	1.95	1.33	2.70	1.52	0.743	0.842	1.64	0.339	1.57	1.55
P_1			0.49	0.16	0.38	0.45	0.079	0.032	0.24	0.016	0.39	0.84
P_2	0.021		0.53		0.127		0.044		0.022		0.52	
Vitamin B_{12} N	13	18					1	11	12	14	7	6
M	323	171					20	222	138	217	266	252
σ	146	169						240	19.9	97.4	22.4	190
P_1								0.67	0.001	0.067	0.34	0.32
P_2	0.017								0.033		0.28	

Symbols: GF, germfree; Conv., polycontaminated (conventional) chicks; P_1, probability for chicks on a complete diet, as compared to those on a deficient diet; P_2, probability for germfree chicks as compared to conventional chicks on the same diet.

TABLE 7
CECAL CONTENTS

Analysis	Complete diet		Diet deficient in									
			Thiamin		Riboflavin		Niacin		Folic acid		Vitamin K	
	GF	Conv.	GF	Conv.	GF	Conv.	GF	Conv.	GF	Conv.	GF	Conv.
Flavin N	3	10	1	3		5	6	6	2	4	5	5
M	10.9	1.38	13.5	4.43		3.3	5.0	9.4	5.4	4.6	3.6	2.6
σ	7.57	2.38		—		0.040	1.04	2.14	1.48	1.50	0.400	0.775
P_1						0.005	0.18	0.70		0.067	0.011	0.001
P_2	0.089						0.004		0.069		0.26	
Niacin N	9	13	1				5	10	1	3	8	6
M	12.1	37.2	64				14.6	37.2	7.8	8.1	25.0	19.1
σ	6.7	18.7					3.80	20.6		2.30	3.00	5.06
P_1							0.83	1.0		0.022	<0.001	0.041
P_2	0.003						0.114				0.11	
Ca pantothenate N	9	12	1			2	1	5		1	4	5
M	5.47	7.74	28.4			8.0	15.2	11.7		2.9	16.2	3.88
σ	2.40	3.12				3.30		1.84			6.30	0.794
P_1								0.19			<0.001	0.046
P_2	0.68										0.016	
Biotin N	9	13	1	3	3	3	2	2			4	4
M	0.241	0.488	3.8	0.15	2.4	0.15	0.21	0.38			0.35	0.49
σ	0.182	1.59			0.373	0.065					0.160	0.285
P_1					<0.001	0.74					0.36	0.9+
P_2	0.664				0.001						0.20	
Folic acid N	7	13	1	3		4	6	7	9	13	5	5
M	2.29	1.83	3.0	0.35		1.1	2.1	0.74	0.55	1.22	1.05	0.67
σ	0.46	0.94				0.575	0.49	0.208	0.123	0.358	0.414	0.083
P_1						0.24	0.74	0.009	<0.001	0.016	<0.001	0.020
P_2	0.255						0.13		<0.001		0.10	

Symbols: GF, germfree; Conv., polycontaminated (conventional) chicks; P_1, probability for chicks on a complete diet, as compared to those deficient diet; P_2, probability for germfree chicks as compared to conventional chicks on the same diet

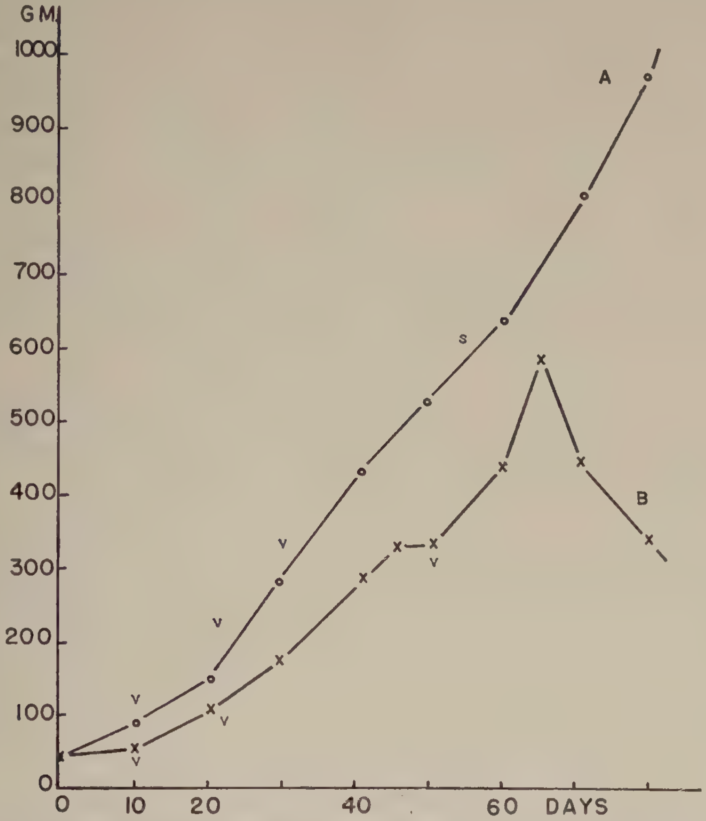

FIGURE 27. White Leghorn chicks fed a purified diet. Symbols: A, germfree birds (9 at eginning of experiment, 1 remaining at conclusion; B, control birds (4 at beginning of experiment, 1 remaining at conclusion); S, *Staphlyococcus* infection; V, vitamin B_1 therapy.

Folic acid deficiency. This condition was readily obtained in 10 germfree hicks. Growth, feathering, and hemoglobin formation were retarded, as vas expected (FIGURE 26). Analysis of the livers of germfree birds (TABLES and 7) indicated a lower concentration of thiamin, pantothenic acid, and itamin B_{12} than was seen in folic acid-fed birds. No differences were noted in he liver content of niacin, biotin, or folic acid. Riboflavin was found to be igher in the liver of deficient germfree birds than in those on the complete iet. When the liver vitamin concentration in deficient birds was compared, ermfree chicks were found to be similar to conventional birds in thiamin, iacin, pantothenic acid, and biotin, and they were found to have more ribolavin and folic acid and less vitamin B_{12} than deficient conventional chicks.

The folic acid concentration in (wet) cecal contents was lower in both germree and conventional deficient birds than in those fed folic acid. There was ess folic acid in the cecal contents of germfree birds than in polybiotic birds, oth on the deficient diet.

Vitamin D deficiency. The growth curves (FIGURE 26) illustrate a dramatic difference in growth between the controls and vitamin D-deficient birds. One normally does not associate a vitamin D deficiency with growth, but here it is quite obvious. Germfree chicks deficient in vitamin D feathered poorly and presented a shabby appearance. Their "hock" joints were enlarged, and the keel bones were usually misshapen. Analysis of these germfree chicks

TABLE 8

PER CENT COMPOSITION OF TISSUES OF CHICKS DEFICIENT IN VITAMIN D

	Vitamin D deficient diet		Complete diet	
	GF	Conv.	GF	Conv.
Liver				
Dry Wt. N	4	4	4	4
M	28.9	28.8	27.1	28.8
σ	1.54	0.818		
P_1	0.53	1	3.20	1.29
P_2	0.71			
Ash N	4	4	4	4
M	6.05	5.74	6.49	5.39
σ	1.53	0.624	0.30	0.34
P_1	0.62	0.63		
P_2	0.75			
N_2 N	3	4	3	4
M	3.34	3.27	3.13	3.46
σ	0.10	0.161	0.20	0.16
P_1	0.91	0.92		
P_2	0.54			
Fat N	3	4	4	4
M	1.85	1.46	5.25	4.07
σ	1.13	0.671	0.27	1.20
P_1	<0.001	0.016		
P_2	0.63			
Muscle				
Dry Wt. N	4	4	4	4
M	20.8	20.0	21.7	23.2
σ	2.42	1.99	0.87	0.73
P_1	0.55	0.046		
P_2	0.67			
Ash N	4	4	3	4
M	7.32	4.86	5.23	4.35
P_1	1.33	0.528	0.26	0.96
σ	0.019	0.45		
P_2	0.073			
Fat N	3	3	3	4
M	2.89	4.55	2.34	2.25
σ	1.42	0.352	0.14	0.17
P_1	0.14	<0.001		
P_2	0.59			

TABLE 8—*Continued*

	Vitamin D deficient diet		Complete diet	
	GF	Conv.	GF	Conv.
	Brain			
Dry Wt. N	4	4	4	4
M	19.3	18.5	16.0	17.8
σ	2.25	4.63	1.50	1.6
P_1	0.076	0.61		
P_2	0.65			
Ash N	3	4	4	4
M	5.17	5.53	12.4	8.0
σ	0.91	0.316	1.2	0.48
P_1	<0.001	<0.001		
P_2	0.53			
N_2 N	4	4	4	3
M	1.63	1.55	1.64	1.73
σ	0.109	0.114	0.14	0.05
P_1	0.46	0.041		
P_2	0.9+			
Fat N	3	1	4	3
M	5.79	6.63	6.79	5.80
σ	0.03		1.2	0.22
P_1	0.26			
P_2				
	Bone			
Dry Wt. N	3	4		
M	40.3	41.3		
σ	6.03	0.314		
P_1				
P_2	0.79			
sh N	3	4	4	4
M	49.9	49.0	66.6	64.6
σ	1.61	2.72	2.72	2.95
P_1	<0.001	<0.001		
P_2	0.68			
at N	3	3	3	3
M	2.08	0.45	21.2	22.0
σ	2.85	0.145	1.06	1.28
P_1	0.001	0.001		
P_2	0.50			

Symbols: GF, germfree; Conv., polycontaminated (conventional) chicks; P_1, probability chicks on a complete diet, as compared to those on a deficient diet; P_2, probability for rmfree chicks as compared to conventional chicks on the same diet.

a complete diet and conventional chicks on each diet is presented in TABLE The liver showed no change in dry weight, ash, or nitrogen content. The er fat of both groups of deficient chicks was reduced to about one third of e values obtained from germfree and conventional chicks on the complete diet. Muscle tissue of vitamin D-deficient germfree birds had the same dry weight and fat content as that of germfree birds on the complete diet and conventional birds on the deficient diet. The quantity of ash in muscle of vitamin D-deficient chicks was greater than that found in germfree birds on the complete diet and tended to be greater than that ash content of muscles from conventional vitamin D-deficient birds. Except that the ash content of the brains of vitamin D-deficient birds, whether germfree or not, was about one half as great as in birds on the complete diet, no differences were seen in the gross composition of brain. Bone tissue showed no differences in dry weight, and the expected decrease in ash content in birds fed no vitamin D was noted. Surprisingly the fat content of the bones of deficient birds was considerably lower than that of bones of birds on a complete diet. No differences were noted between the fat content of bones of germfree and conventional birds.

Vitamin K. A vitamin K deficiency was obtained in both germfree and conventional chicks, as shown by prolonged clotting times; under both conditions the clotting times of surviving chicks returned to normal.[27] Analysis of livers of chicks on the vitamin K-free diet (TABLES 6 and 7) showed that the concentrations of thiamin, riboflavin, and pantothenic acid were lower in germfree chicks on the deficient diet than in those on the complete diet. The reduction in pantothenic was of considerable magnitude. Niacin, biotin, folic acid and, probably, vitamin B_{12} were similar in both groups. Of these vitamins, only in niacin was a difference found between germfree and conventional chicks on the vitamin K-deficient diet; livers from germfree chicks had less niacin than was found in polybiotic chicks.

No difference was found in the riboflavin or biotin concentration of the cecal contents (on a wet weight basis) between vitamin K-deficient and control germfree chicks. More niacin and pantothenic acid were present in the cecal contents of germfree chicks on the vitamin K-deficient diet than in cecal contents of chicks on the complete diet. The pantothenic acid concentration in cecal contents was also considerably higher than that found in conventional chicks on the vitamin K-deficient diet. The folic acid concentration of cecal contents was lower in germfree chicks on the vitamin K-low diet than in chicks on the complete diet. Riboflavin, niacin, biotin, and folic acid were present in similar quantities in the cecal contents of germfree and conventional chicks.

Monocontaminated chicks. Several opportunities to compare chicks in a dibiotic system to those in a monobiotic (germfree) condition were available during the course of the above work. One of the vitamin K experiments (diet L-258) was found to be contaminated with *Streptococcus fecalis*, an organism that requires as nutrition most of the B vitamins.[28] Analysis of the liver and cecal contents of dibiotic chicks harboring only this organism with that of a monobiotic chick revealed no difference consistent with the idea that the organism was metabolizing excess quantities of the vitamins it required (TABLE 9). The cecal contents of the monocontaminated birds contained more riboflavin, less niacin and folic acid, and about the same concentrations of pantothenic acid and biotin as were found in the cecal contents of monobiotic (germfree) birds. The concentrations of all vitamins were similar to those in the two control (polybiotic) birds tested at the same time. It is

TABLE 9

VITAMIN ANALYSIS OF CHICKS FED L-258

No. chicks	Liver μg./gm. Thiamin	Riboflavin	Niacin	Ca ponto-thenate	Pyridoxine	Biotin	Folic acid	APF (B_{12}) μg./gm.	Cecum μg./gm. Riboflavin	Niacin	Ca ponto-thenate	Biotin	Folic acid
ermfree (3)...	4.53	19.0	117	15.3	11.5	6.5	2.7	266	0.73	29.2	16.2	0.35	8.2
ontrol (2).....	4.98	15.3	132	16.1	11.2	4.5	1.7	156	—	16.5	—	0.20	—
F (4).........	4.39	26.8	12.7	26.2	—	4.4	1.3	—	13.3	2.5	18.1	0.47	3.2
ontrol (2).....	4.77	23.5	319	20.4	--	3.9	1.2	—	10.5	3.1	12.6	1.40	1.2

TABLE 10

ANALYSIS OF MONOBIOTIC, DIBIOTIC, AND POLYBIOTIC (CONVENTIONAL) CHICKS

	Liver				Cecal contents		
	No. chicks	Biotin	Folic acid	B_{12}	Biotin	Folic acid	B_{12}
ermfree*.....	7 to 13	5.14	4.41	0.32	0.24	2.29	0.015
3-32 *Aerobacter aerogenes*.. ..	2	4.01 3.89/4.14	3.80 3.50/4.09	0.33 0.33/0.33	0.436 0.457/0.478	— —	0.057
3-41 *Pseudomonas jaegeri*.	3	5.64 5.08/6.06	4.66 2.88/8.18	0.15 0.13/0.16	0.15	2.12	0.019
onventional...	3	7.35 4.11/10.3	3.98 3.51/4.31	0.10 0.09/0.11	0.21	1.87*	0.017*

* These values taken from other experiments. All chicks on diet L-245.

nfortunate there were not more birds in each group, and that tests on the ermfree and dibiotic birds were not done at the same time, so that a more alid comparison could be made. Monocontaminated chicks having a single pecies of a Gram-negative microorganism as the other member of the dibiotic ystem were also studied (TABLE 10). Both of the organisms in question are resumed to synthesize most B vitamins.[29] With the possible exception of he vitamin B_{12} and biotin concentrations of the cecal contents of chicks ontaminated with *Aerobacter aerogenes*, biotin, folic acid, and vitamin B_{12} alues were not higher than those found in germfree chicks. Again, the small umber of samples would not validate any differences obtained, so this must e considered little more than a pilot study done as the opportunity presented tself.

Antibiotic effects in germfree birds. The effect of antibiotics upon the growth f germfree and conventional birds on practical diets has been presented elsewhere in detail[30] and has been evaluated in the light of other relevant work.[31] t may be summarized (FIGURE 28) by pointing out that the growth increase

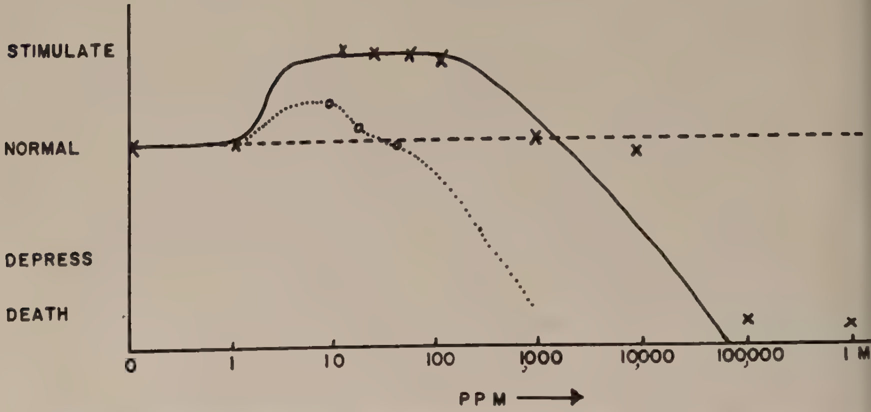

FIGURE 28. Activity spectrum of terramycin as a hormoligant.* Dotted line, germfree chicks; solid line, conventional chicks.

* A hormoligant is any substance or physical effect that will produce a stimulatory effect when administered in minute amounts and that is harmful when given in large quantities.

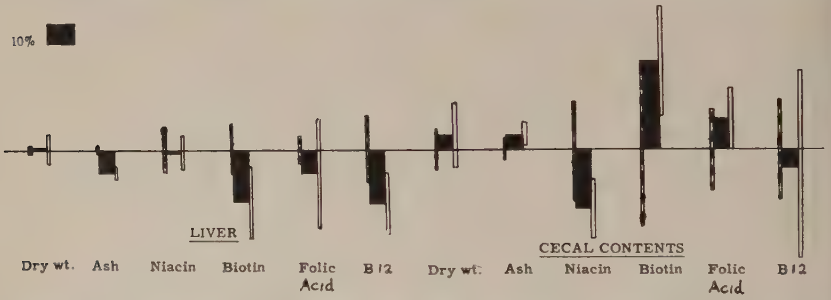

FIGURE 29. Analysis of the germfree chick with and without oxytetracycline in the diet. The base line represents the values for chicks fed the practical diet without the drug; the heavy bar indicates the difference on a percentage basis; the narrow bars show the coefficient of variability as a percentage of the mean; the hatched bar shows the untreated chicks; and the open bar shows the variation obtained from chicks fed the drug.

observed in conventional animals using a relatively large amount of antibiotics, caused to a large extent by the antibacterial activity of the drugs, is obviously not seen in germfree animals. In germfree animals a positive growth response is obtained only when very small quantities of the drug are fed. Lack of appreciation of the principle of hormoligosis* made our original survey appear contradictory and caused others to miss the effect entirely.

The effect of antibiotics upon the vitamin contents of liver and cecal contents was studied in limited numbers of chicks. The data (FIGURES 29 to 32) are presented with the untreated animal represented by the base line, and the change found in chicks fed the antibiotic is given as a bar that represents the precentage difference from the control. The coefficient of variability of the

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* Hormoligosis is the stimulatory action of a minute quantity of a harmful material.[32] See also Forbes *et al.* elsewhere in this monograph.

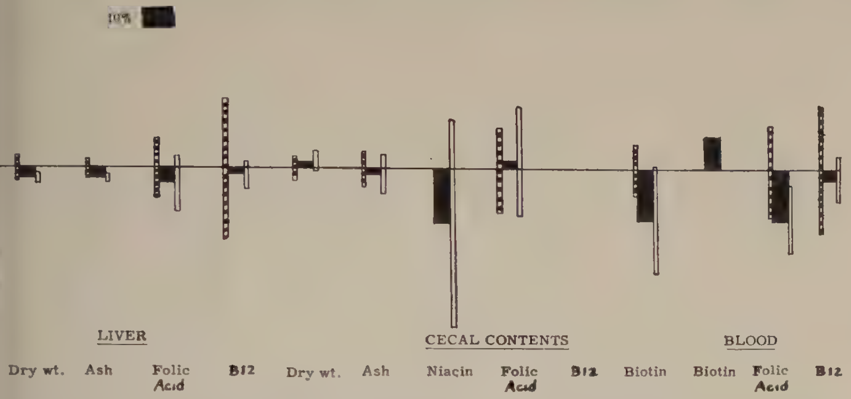

FIGURE 30. Analysis of germfree chicks with and without streptomycin in the diet. The ase line represents the values for chicks on the practical diet without the drug; the heavy ar indicates the difference on a percentage basis; the narrow bars show the coefficient of ariability as a percentage of the mean; the hatched bars show the untreated chicks; and the pen bar shows the variation obtained from chicks fed the drug.

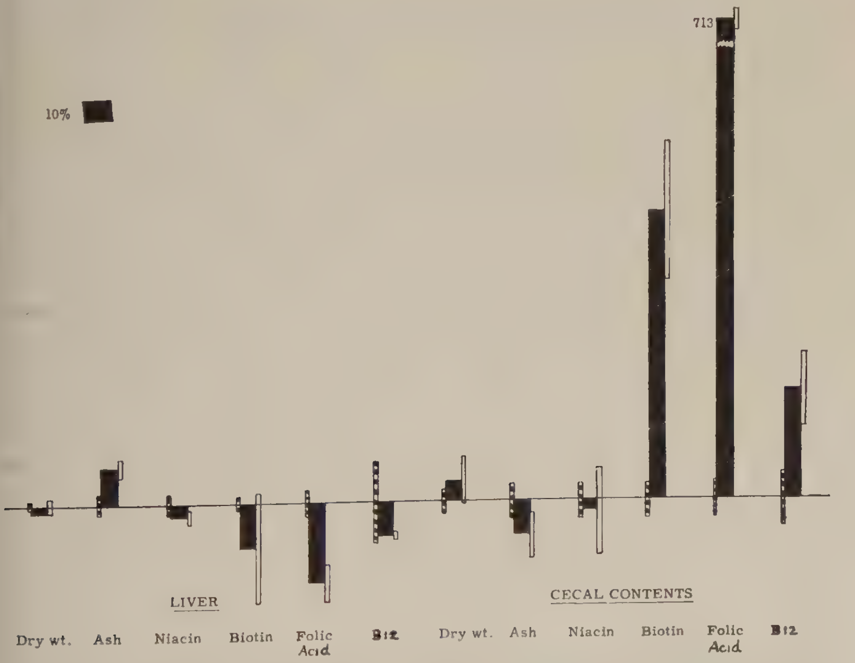

FIGURE 31. Analysis of conventional chicks with and without oxytetracycline in the diet. The base line represents the values for chicks on the practical diet without the drug; the heavy bar indicates the difference on a percentage basis, the narrow bars show the coefficient of variability as a percentage of the mean; the hatched bars show the untreated chicks; and the open bars shows the variation obtained from chicks fed the drug.

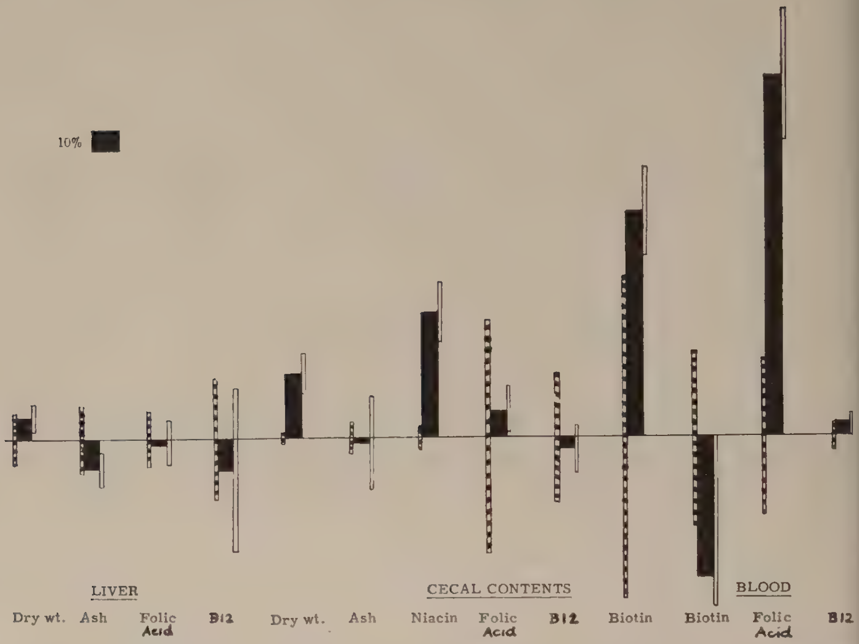

FIGURE 32. Analysis of conventional chicks with and without streptomycin in the diet. The base line represents the values for the chicks on the practical diet; the heavy bar indicates the difference on a percentage basis; the narrow bars show the coefficient of variability as a percentage of the mean; the hatched bars show the untreated chicks; and the open bars show the variation obtained from chicks fed the drug.

chicks fed no antibiotic is given by the narrow hatched bar at the left of each value, and that for the treated chicks is given by the open narrow bar at the left of the main bar. Each value represents 3 to 7 chicks per analysis. Fifty-mg. doses of oxytetracycline or streptomycin produced no significant differences in the concentration of vitamins in liver or cecal contents of germfree birds.

Conventional birds fed oxytetracycline showed high liver ash, generally lower B-vitamins in the liver, and some very high biotin, folic acid, and vitamin B_{12} concentrations in the cecal contents. When streptomycin was fed to conventional chicks the liver constituents were relatively constant, while the blood folic acid content was high, and the cecal contents had more dry matter, more niacin, and more biotin as compared to conventional chicks on the same diet without antibiotics.

Discussion

The reason for using the germfree animal as the base line in much of this work is that the microflora represented an added variable to the system. Surprisingly, birds in a polybiotic system do not appear to show greater variation in the results obtained. If the coefficient of variability is compared for the germfree and conventional birds, it is noted in a hundred comparisons that either group is substantially more stable, from the chemical viewpoint, than he other. Elsewhere in this monograph Gordon suggests that some organs of onventional chicks may be less stable morphologically than those of germfree hicks. If this be true, the tissue (lymphatic, for example) causing variation s apparently of similar gross composition to that of the parent organ. Pos-ibly certain tissues of the germfree chick develop more slowly due to less ntigenic stimulation, and thus appear to be more stable because they have een examined at an earlier time from a developmental viewpoint, although hronologically the chicks compared are the same age.

The pattern of vitamin metabolism in different tissues under different dietary tress situations should be considered. In TABLE 5 we note that thiamin and iboflavin concentrations in the liver change with almost every vitamin de-ciency imposed upon the chick. Niacin and pantothenic acid concentrations hange for about one half of the deficiencies imposed, while folic acid and articularly biotin appear to be stable components of liver tissue, irrespective f the state of the chick.

Examination of chicks on complete syntype diets from the same viewpoint FIGURES 11 to 22) indicates less stable components of tissue (with respect to he presence or absence of the microflora) are moisture, ash, vitamin C, and antothenic acid. The liver tends to be the least stable tissue examined. Bile lso varied more than had been anticipated. It is of interest to note that in all f the significant differences in B vitamin content of tissues (excepting the oncentration of niacin in cecal contents) the conventional birds had lower oncentrations than the germfree birds. Speculation to extrapolate such dif-erences into coenzyme or enzyme concentrations might extend as far as coenzyme A in those instances (liver and brain tissue) where pantothenic acid was higher in germfree birds.

This summary represents the main body of published knowledge with respect to the biochemistry of germfree vertebrates. More may be gleaned from the present symposium and from the literature on rats and goats. This view of the nutrition of germfree chicks represents a sizeable fragment of what is known regarding the nutrition of germfree vertebrates. However, an equal amount of data is found on mammals that should be considered in any thorough examination. Since many of these data are being presented for the first time in this monograph, it is premature to attempt a critical evaluation of them here. In general, it appears that results obtained with the chick indicate the main pattern of the results obtained with mammals. Differences in some details have already appeared, such as the apparent requirement for folic acid by the germfree chick and its synthesis by the germfree rat.[33]

Summary

The biochemical implications of germfree research, initiated by Pasteur n the last century, have come into experimental fruition only in the past decade, while nutrition has been, historically, the principal problem in the successful rearing of animals in the germfree state. At present the vitamin and protein losses incurred during sterilization of the diets are made up by adding excess quantities of the labile nutrients. Diet may be sterilized by steam under pressure in the liquid or dry states. Chicks fed a liquid diet become immobilized by the gluelike material. Supplements for diets may be sterilized by filtration. Gamma-ray sterilization is very useful, and ethylene oxide (as carboxide) has been used to sterilize diets for chicks. Germfree chicks have also been used as feed for germfree rats.

Monobiotic chicks grow satisfactorily when fed either practical or syntype diets that are complete after sterilization. The chemical composition (vitamin and gross analysis) of the chicks is generally similar to that of polybiotic chicks. The differences observed indicate that liver and bile are less stable biochemically than other tissues examined. Folic acid and particularly biotin are the most stable vitamins in tissues of chicks subjected to dietary stresses of vitamin deficiencies.

Germfree chicks were found to require the following vitamins: A, D, E, K, thiamin, riboflavin, niacin, biotin, and folic acid. Studies with other B vitamins were inconclusive. The interrelationships of vitamins was studied by analyzing tissues of chicks in different deficiency states.

Comparisons were made, as opportunity allowed, between monobiotic chicks and chicks in different dibiotic (harboring a single species of microorganism) systems.

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VITAMIN K DEFICIENCY IN GERMFREE RATS*

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The intestinal synthesis of vitamins has recently been reviewed by Mickelsen (1956), and there is ample evidence that, under ordinary conditions, intestinal microorganisms are able to supply a considerable amount if not all of the required amount of vitamin K. It is, then, astonishing that studies of vitamin K deficiency in germfree animals have given conflicting results. Gustafsson (1948) reported the deaths of weanling germfree rats, with bleeding tendency and impaired clotting of the blood. Signs of disturbances in the blood-clotting mechanism in germfree rats were also observed in studies on liver necrosis by Luckey *et al.* (1954). These signs, however, could be partially prevented by vitamin E. When germfree chicks were maintained by Luckey *et al.* (1955) on a diet deficient in vitamin K, the blood-clotting times increased for one week, but then returned to normal for the remainder of the experimental period of 24 days. Moreover, Luckey *et al.* (1955) were unable to produce prolongation of the blood clotting time in germfree rats on a vitamin K-deficient diet for 400 days.

In view of these conflicting observations the purpose of the present investigation has been to study the effect on germfree animals of the withdrawal of vitamin K from the diet.

Methods

Rats of the germfree colony of the Long-Evans strain reared according to Gustafsson (1948, 1958) were given a vitamin K-deficient diet (TABLE 1). This diet is basically the same as is generally used for rearing the germfree colony with vitamin K omitted, however, and the casein replaced by a commercial brand of vitamin-free casein. In some series the casein and starch were extracted with petroleum ether. The study comprised 44 germfree rats and 29 conventional rats of both sexes. The two groups of rats were kept on raised screens in the germfree apparatus and in the animal room, respectively. The diet, sterilized by autoclaving for 30 min., and water were consumed *ad libitum.*

In some series the diet was given to the pregnant females that later delivered the experimental animals. In two other series the animals received the diet at 1 and 2 months of age, respectively, after having been weaned to the standard vitamin K_1-containing diet.

Prothrombin levels were determined as the prothrombin time according to Quick's method as modified by Lehmann (1941), using human brain thromboplastin. The values were calculated as percentages of the normal content corresponding to a mean prothrombin time of 20 seconds in stock rats, using the same human brain thromboplastin preparation.

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TABLE 1

VITAMIN K-DEFICIENT DIET

Casein, "vitamin free"*	22%	
Wheat starch*	63%	
Peanut oil	10%	
Salt mixture HMW†	4%	
Vitamin mixtures	1%	
Vitamins added per 100 gm. diet:		
Vitamin A	2100	I.U.
Vitamin D	450	I.U.
Vitamin E	50	mg.
Thiamine	5	mg.
Riboflavin	2	mg.
Pyridoxine	2	mg.
Calcium pantothenate	10	mg.
Nicotinamide	20	mg.
Choline	200	mg.
Inositol	100	mg.
p-Aminobenzoic acid	30	mg.
Biotin	0.1	mg.
Folic acid	2	mg.
Vitamin B_{12}	0.002	mg.
Ascorbic acid	100	mg.

* Extracted with petroleum ether.

† According to Hubbell *et al.* (1937).

Results

TABLE 2 shows that all of the 44 germfree rats developed hypoprothrombinemia, 42 rats having severe deficiency symptoms either with bleeding tendency or prothrombin values below 10 per cent (prothrombin times above 200 seconds). In some of these animals the blood did not coagulate even after several hours. At autopsy 35 of the germfree rats had hemorrhages, very severe in some cases, and 12 animals succumbed during the experiments.

The 29 control rats on the sterilized vitamin K-deficient diet had prothrombin times that were the same as, or shorter than, those of the stock rats. None of these animals showed hemorrhages, and there were no fatalities.

The first hemorrhages were encountered at 26 days of age in 18 animals born

TABLE 2

FREQUENCY OF DEFICIENCY SYMPTOMS IN GERMFREE AND CONTROL ANIMALS ON A VITAMIN K-DEFICIENT DIET

Number of animals	Germfree	Control
Total on diet	44	29
With hypoprothrombinemia	44	0
With bleeding tendency or prothrombin below 10 per cent	42	0
With bleeding tendency	35	0
Dying	12	0

to mothers on the deficient diet, that is, 5 days after weaning. In 12 animals turned over from a vitamin K_1-containing diet to the deficient diet at 30 and 60 days, respectively, a bleeding tendency occurred after 7 to 9 days. In these three series, in each of which the animals showed symptoms within 9 days, the casein and starch of the diet were extracted. When this operation was not performed, a bleeding tendency was not noticed until after 22 days in the group of 14 animals given the diet at the age of 30 days. In this latter group there was also 1 rat with a prothrombin value above 50 per cent.

As soon as the first signs of bleeding tendency were encountered the animals were used for special studies (see below) or sacrificed to procure material for histological examinations. The corresponding control animals of the series were sacrificed at the same time.

In some animals the hemorrhages were extensive. The distribution is interesting, as 19 of the 35 rats with bleeding tendency had hemorrhages in the thymus (FIGURE 1), which was sometimes so greatly distended that it filled more than one half of the thoracic cavity, dislocating the heart and lungs. This was probably the reason for the labored breathing seen in the animals having an enlarged thymus.

Cephalic hematomas of a clinical and pathological pattern very similar to those in newborn children occurred in 13 of the 35 animals with hemorrhages (FIGURE 2). In the subcutaneous connective tissue hemorrhages were seen outside the parietal region in 9 animals. Muscles, testes, and intestines were the sites of bleedings in 7, 5, and 2 animals, respectively.

With the exception of the labored breathing described above and the swellings due to subcutaneous hemorrhages, the animals were without symptoms,

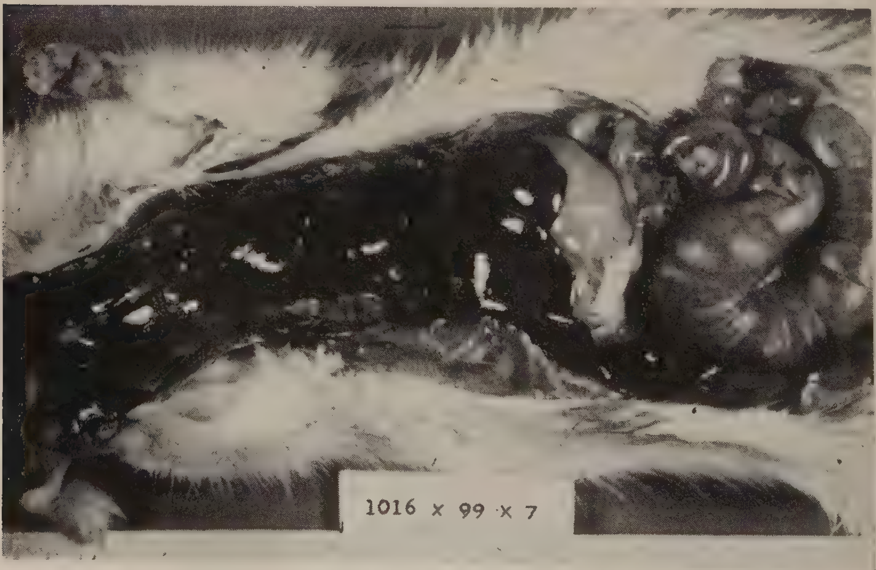

FIGURE 1. Hemorrhage of the thymus in a germfree rat with vitamin K deficiency.

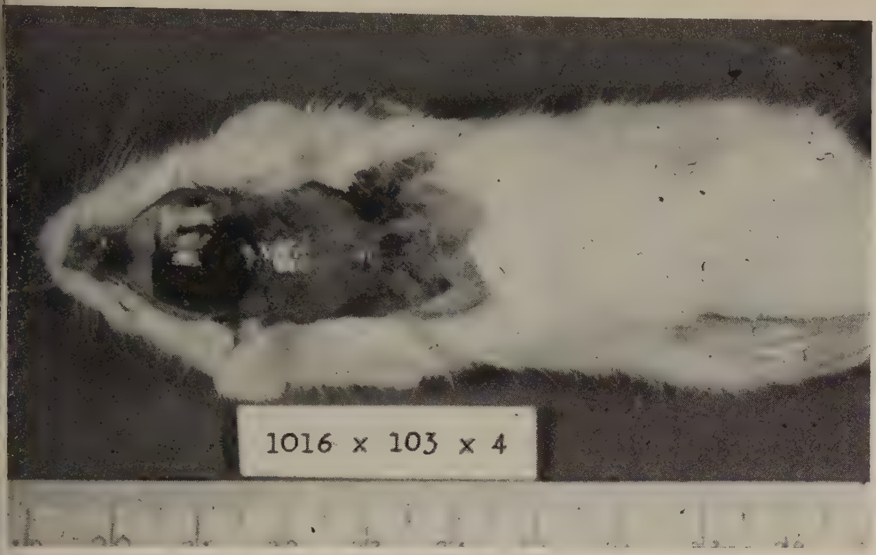

FIGURE 2. Cephalic hematoma in a germfree rat with vitamin K deficiency.

appeared remarkably healthy, and seemed lively. In many cases, the rats remained so until a very short time before death.

Treatment Experiments

To ensure that the germfree state was the reason for the difference between the germfree and control groups, animals with prothrombin values at or below 10 per cent were transferred from the tanks to the laboratory without changing the diet. To demonstrate the influence of the flora, some of these vitamin K-deficient animals were placed in heavily infected surroundings, that is, in cages that had been occupied just previously by the control animals. As the cages were not washed, there was a possibility that the animals were able to consume small amounts of fecal material by licking the screen. A second group of germfree, vitamin K-deficient animals was moved to cleaned metabolism cages in the animal room, that is, to a moderately infected area. A third group of germfree K-deficient rats was transferred to a laboratory well away from the animal room and kept in glass jars or stainless steel boxes that had been washed with a germicide. The prothrombin times were determined on blood samples from the tails every 24 hours. Each of the 3 experiments showed the same primary result. FIGURE 3 gives the results for 1 of the experiments. The animals in the heavily infected surroundings had normal prothrombin times within 48 hours, whereas those in contact with surroundings with presumably less bacteria and, possibly, a flora of quite another type showed only a slight change within 72 hours. Rats in the moderately infected area formed an intermediate group.

The administration of drugs and the repeated taking of blood samples were rather difficult in the smaller types of tanks used in these studies. The following experiments, in which we attempted to remedy the vitamin deficiency, were therefore performed on germfree, vitamin K-deficient animals from groups in which a bleeding tendency had developed. These animals were then transferred from the germfree tank to the glass jars and stainless steel boxes described, where the probability of recovery due to an established flora might be slight within the first 24 hours.

In the first experiment 2 animals of the same litter and with almost the same prothrombin times were transferred to the laboratory. The animal with the lowest prothrombin value was given a dose of water-soluble vitamin K_1 equivalent to 1 mg./kg. body weight by stomach tube immediately after the transfer. The prothrombin time was determined every hour in each animal. FIGURE 4 demonstrates that there was a very rapid increase of the prothrombin

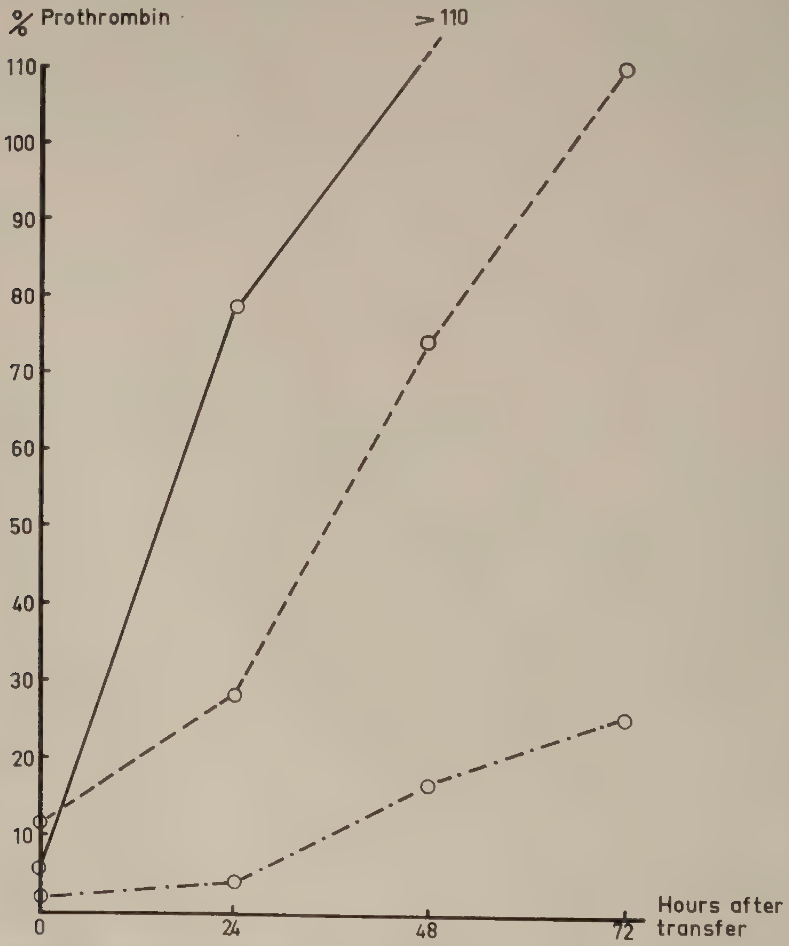

FIGURE 3. Prothrombin values in germfree rats with vitamin K deficiency transferred to a heavily infected ordinary cage (O——O); to a moderately infected metabolism cage (O------O); and isolated in glass jars in the laboratory (O-·-·-·O).

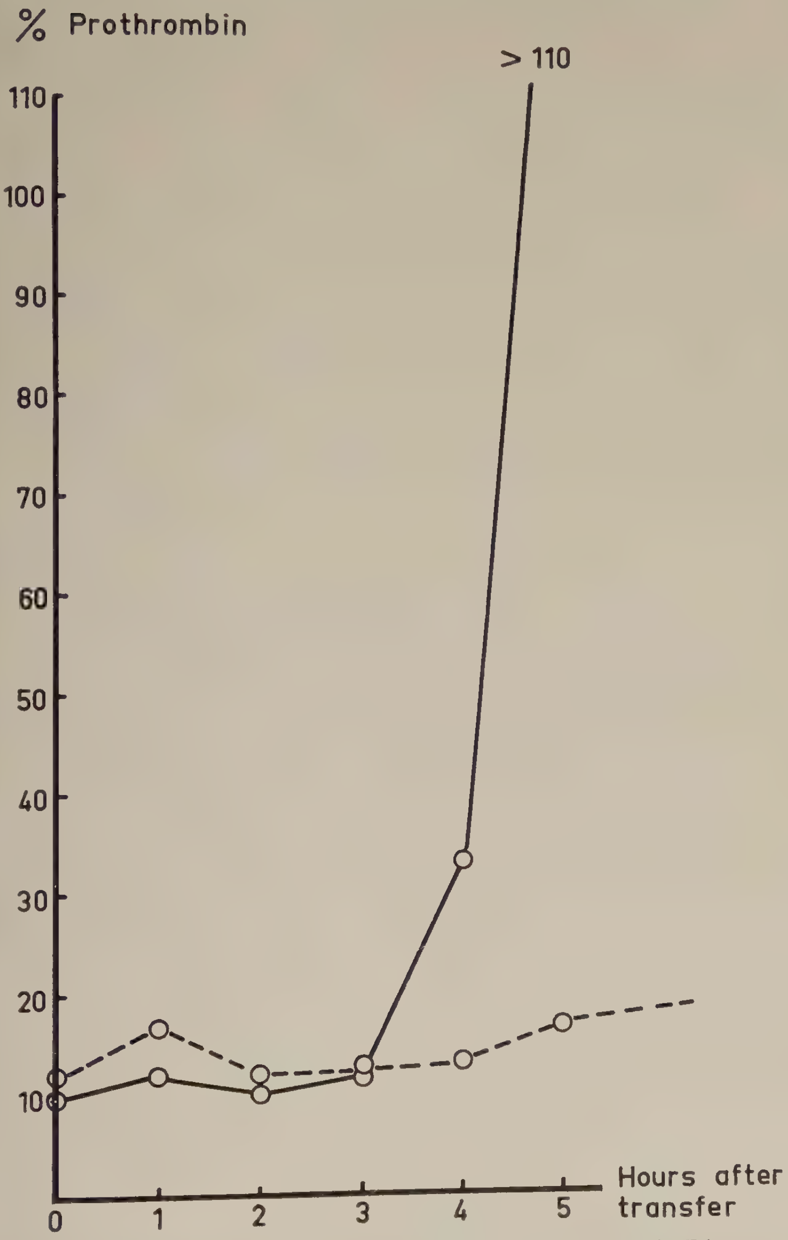

Figure 4. Prothrombin values after a single oral dose of 200 μg. vitamin K in a germfree rat with vitamin K deficiency (○——○) and of a littermate given sodium chloride solution (○------○) at 0 hours.

value after 3 hours, and that the value was normal within 5 hours. The other animal, which received the same amount of fluid as the littermate, was given physiological saline by means of a stomach tube and showed only small fluctuations within the first 5-hour period.

In similar experiments on a limited number of animals with a prothrombin value below 10 per cent the efficiencies of sodium menadione sulfate, sodium menadione phosphate, and fat-soluble 2,3-methyl-1,4-napthohydroquinone were compared with that of vitamin K_1. The compounds were given by stomach tube in amounts equivalent to 1 mg./kg. body weight. After 8 hours no change was found in the prothrombin levels of the 6 rats that had received physiological saline, sodium menadione sulfate, or 2,3-methyl-1,4-napthohydroquinone. The two rats given sodium menadione phosphate had respective values of 57 and 71 per cent after 8 hours, whereas at the same time the 3 rats given vitamin K_1 all had normal prothrombin times. At 16 hours the rats given the menadione phosphate still had somewhat subnormal values (75 and 90 per cent). The control rats and those given menadione sulphate and the fat-soluble menadione had not increased their prothrombin times above the 10 per cent level at 16 hours after transfer from the germfree tanks.

Discussion

The germfree animals on the vitamin K-deficient diet showed greatly prolonged prothrombin times and a bleeding tendency within the rather short time of 5 to 9 days, whereas the control conventional animals on the same diet still had normal prothrombin values after 24 days and no tendency to bleed. This difference is probably due to the absence of bacteria in the germfree animal. This and the facts that the deficient animals were cured by transfer to an infected area and also cured very rapidly by the administration of vitamin K_1 form strong evidence for the long postulated concept that vitamin K is synthesized by the microorganisms normally present in the intestines of mammals. This synthesis is capable of supplying the total need of the animals under ordinary conditions. Whether the vitamin is absorbed directly or after coprophagy is still debatable. Barnes and Fiala (1958) have recently demonstrated that by complete prevention of coprophagy in weaned rats receiving a vitamin K-deficient diet, 100 per cent of the animals showed prothrombin values lower than 17 per cent within 28 days. Many of them showed bleeding tendencies.

In some of our series the vitamin K-deficient diet was prepared with casein and starch that had not been extracted with petroleum ether. The onset of symptoms of bleeding tendency was much delayed, that is, until the twenty-first day. Discrepancies in the preparation of the diets or profound differences among species might explain the controversial finding by Luckey and his collaborators that germfree chicks and rats do not develop vitamin K deficiency when vitamin K is omitted from the diet.

The results on the curative efficiency of menadione compounds must be considered to be only preliminary. These experiments are fraught with the difficulty that the effect could not be followed for more than 16 hours. Later the developing bacterial flora might have influence under the experimental conditions used. The negative result with sodium menadione sulfate is, however, in accordance with the findings in earlier series of our germfree rats reared to work out the semisynthetic diet. A large number of both suckling and adult rats showed prolonged clotting times and died with hemorrhages, although 2 mg. sodium menadione sulfate was supplied per 100 gm. of diet.

Summary

On a semisynthetic diet containing vitamin-free casein and starch and with all the known vitamins except vitamin K_1 added, 42 of 44 germfree rats developed signs of severe hypoprothrombinemia. Thirty-five rats had bleeding tendency, and 12 died. The disturbances of the blood-clotting mechanism could be alleviated by transferring the animals from the germfree tanks to more or less heavily contaminated surroundings. After oral administration of vitamin K_1 the prothrombin values below 10 per cent also returned to normal within 5 hours.

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Discussion of the Paper

Question: There is a theory that vitamin K must be converted to a natural form of the vitamin before it can be utilized in normal blood-clotting mechanisms with respect to the prothrombin level. Vitamin K_3 cannot be utilized in this manner. Have you any explanation for the fact that menadione sulfate gave a negative result while menadione phosphate gave a positive result?

Gustafsson: I can offer no suggestion other than that there must be some difference in the rate or manner of absorption?

Question: Then we must still assume that menadione phosphate as such acted by being converted to natural vitamin K?

Gustafsson: On the basis of the present data I believe this to be the case. In this connection I point to the well known fact that vitamin K_1 was much more effective than menadione in relieving the bleeding tendency of dogs intoxicated by Dicumarol. In some of these dogs menadione had no effect at all. The theory has been proposed that Dicumarol acts by preventing a supposed conversion by intestinal bacteria of menadione to a natural form of vitamin K. Although this possibility is rather remote, we administered Dicumarol to a limited number of germfree and conventional rats. The germfree rats developed a bleeding tendency at the same time and to the same degree as the conventional rats.

NUTRITION OF THE GERMFREE MAMMAL*

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The main difficulty facing the nutritionist when working with germfree animals is that, while much may be known about the requirements of the host-flora complex, virtually no quantitative knowledge is available about the nutritional requirements of the host per se. Qualitatively speaking, the nutritional requirements of germfree animals do not seem to differ much from those of their conventional counterparts, although one might expect a requirement for certain nutritional factors normally provided by the bacterial flora. Biotin seems to be such a factor in the rat.[1] Digestive and adsorptive systems of germfree and conventional animals seem more or less comparable. Work done by Lepkovsky[2] on intestinal trypsin, lipase, and amylase, and by Larner and Gillespie[3] on carbohydrases did not indicate any important differences. If any, trypsine activity in the germfree animal (as observed in rats and chickens) seemed somewhat increased. Data obtained at the Lobund Institute by Csaky[4] seem to show that the germfree rat manifests a somewhat faster disappearance of glucose and 3-methyl-glucose from the intestinal tract.

In composing diets for germfree animals, this is all the information available. It will be necessary to use the known requirements of the normal stock animal as a base line. In using these, correction must be made for the absence of possible intestinal synthesis (mostly of vitamins) and for the effects of the necessary sterilization, which results in loss of vitamins and of nutritional value of protein. In particular the sterilization of the diet poses a question of its own. With the steel equipment used thus far by most investigators, steam sterilization (123° C., 17 lb. for 25 min.) has proved to be the safest procedure. However, the rapid development of plastic isolators may shift the emphasis partially to radiation sterilization, which already has proved its usefulness.[5] The possibility of using chemical sterilization, such as the use of ethylene oxide, at this stage seems questionable, to say the least.[6-8]

In both steam sterilization and radiation sterilization of diets, the various vitamins, especially thiamine, are the most vulnerable components. As indicated above, steam sterilization may cause losses of dietary and added thiamine of 80 to 90 per cent. Losses of other vitamins mostly do not exceed 40 to 50 per cent. Steam also affects the nutritional value of the dietary protein, especially affecting the availability of lysine.

In radiation sterilization, at the doses necessary for complete sterility (min. 2×10^6 rep†; in our experiments an average of 2.8×10^6 rep) the nutritional value of proteins may be considered hardly affected.[9] Fortification of the normal formulas, especially when steam sterilization is used, therefore seems a

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† Roentgen equivalent physical.

necessity. However, it should never be overlooked that this may lead to difficulties. Let us assume that the young normal stock rat (100 gm. body weight) needs a dietary intake of 20 μg. of thiamine per day, incorporated in 10 gm. diet, which calls for 0.2 mg./100 gm. of diet. Correcting for the absence of possible flora-synthesized thiamine, its germfree counterpart may need a dietary intake of 40 to 50 μg./day, corresponding to 0.4 to 0.5 mg./100 gm. diet. A possible loss of as much as 90 per cent due to steam sterilization increases the required concentration by a factor of 10 or to 4 to 5 mg./100 gm. diet before sterilization. Steam sterilization then destroys approximately 4 out of the 5 mg., which means that for each milligram of available thiamine, 4 mg. of degradation products are formed. The loss of the NH_2 group seems to be one of the primary occurrences in the degradation of thiamine.[10] Thus, upon sterilization the formation of oxythiamine, a notorious antivitamin, may occur. For this reason the fortification of diets, especially with added thiamine, must be regarded with a certain suspicion. Obviously much more work will be necessary before a full evaluation can be made of the effects of sterilization on our experimental diets.

Nevertheless, although the difficulties and uncertainties are still manifold, in certain instances workable solutions have been reached. The diets of various germfree species, because of the cesarean operation and hand-feeding period involved, fall into two groups: first, milk formulas to cover the period until weaning and, second, solid postweaning diets.

Steam Sterilized Solid Diets in the Postweaning Period

Rats. We now use two diets, one semisynthetic (L-356), developed by T. D. Luckey while at Lobund Institute,* and a practical diet (L-462). The semisynthetic diet is essentially an experimental one; changes in its components are easily made, and it has a comparatively low antigenicity. It is a diet based on rice flour, casein, yeast extract, liver powder, corn oil, salts, and added vitamins.[3] L-462, a more practical diet, is composed of wheat flour, corn meal, whole milk powder, milk proteins, alfalfa, and liver, with vitamins and some salts added (TABLE 1). Both diets, in sterilized form, induce a weight gain of as much as 40 gm./week when fed to young normal stock male rats of the Lobund strain. In germfree animals early weight gain of males in our present environment on both diets is approximately 80 per cent of the gain seen in the normal stock, while in females a slight growth reduction is noticeable. Eventually, however, at the age of about one year, the germfree males seem to reach nearly the same maximum weight as do the normal stock animals. Reproduction is comparable to results seen in the normal stock on a commercial rat diet. About 80 per cent of the young are weaned in good health at about 21 days.[11]

With rats, as well as with Swiss mice, using diet L-356, we are now past the tenth germfree generation. No cumulative signs of deficiencies have appeared thus far. The life span of germfree rats seems to be at least comparable to that seen under normal circumstances. The distended cecum, seen to date

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TABLE 1

COMPOSITION OF PRACTICAL RAT DIET L-462

Constituent	Amt./110 gm.
Distilled H_2O*	15 cc.
Whole wheat flour	30 gm.
Corn meal, yellow	32 gm.
Lactalbumin, Sheffield	10 gm.
Casein, Sheffield new process	5 gm.
Whole milk powder	10 gm.
Alfalfa leaf meal	2 gm.
Desiccated liver	2 gm.
$CaCO_3$	0.5 gm.
B mix-75	
Thiamin hydrochloride	3 mg.
Riboflavin	1.5 mg.
Nicotinamide	2.5 mg.
Nicotinic acid	2.5 mg.
Calcium pantothenate	15.0 mg.
Choline chloride	100.0 mg.
Pyridoxine hydrochloride	1.0 mg.
Pyridoxamine dihydrochloride	0.2 mg.
Biotin	0.05 mg.
Folic acid	0.5 mg.
Para-aminobenzoic acid	2.5 mg.
0.1 Per cent trituration B_{12} in manitol	12.5 mg.
Cornstarch	108.75 mg.
Ladek-36	
Vitamin A concentrate, natural ester form	800 I.U.
Vitamin D_3†	100 I.U.
Vitamin E, mixed tocopherols	37.5 mg.
Vitamin K_3, menadione	10.0 mg.
Corn oil	1.958 gm.
Salts-17	
NaCl	299 mg.
KI	4.5 mg.
$MgSO_4 \cdot 7H_2O$	449 mg.
$MnSO_4 \cdot H_2O$	57.7 mg.
$Fe(C_6H_5O_7)_2$	449 mg.
$CuSO_4 \cdot 5H_2O$	22.4 mg.
$CoCl_2 \cdot 6H_2O$	3 mg.
$ZnSO_4 \cdot H_2O$	3.8 mg.
NaF	1.5 mg.
$Na_2B_4O_7 \cdot 10H_2O$	3 mg.
$AlK(SO_4)_2 \cdot 12H_2O$	4.5 mg.
MoO_3	3 mg.
Analysis‡	
N × 6.25	24 per cent
Fat	8 per cent
Ash	3.5 per cent

* Added to facilitate pelleting.

† Delsterol, produced by E. F. du Pont de Nemours & Co., Inc., Wilmington, Del.

‡ After pelleting and steam sterilization.

TABLE 2
COMPOSITION OF RABBIT DIET L-461B-E1

Constituent	Amt./100 gm.
Purina rabbit chow, ground	62.16 gm.
Casein, Sheffield new process	7.0 gm.
Ground bran	30.0 gm.
BC mix-45	
Ascorbic acid	35.0 mg.
i(-)Inositol	35.0 mg.
Riboflavin	0.7 mg.
Calcium pantothenate	3.5 mg.
Niacinamide	3.5 mg.
Pyridoxine hydrochloride	0.7 mg.
Biotin	0.0175 mg.
Folic acid	0.7 mg.
Thiamin hydrochloride	1.75 mg.
Vitamin B_{12}, 0.1 per cent in mannitol	0.7 mg.
Choline chloride	70.0 mg.
Cornstarch	198.43 mg.
Ladek-3	
Vitamin A concentrate, natural ester form	280 I.U.
Vitamin D*	35 I.U.
Mixed tocopherols (1 gm. = 253 I.U.)	52.5 mg.
Menadione	3.5 mg.
Corn oil	700 mg.
Mineral composition (calculated)	
Ca	980 mg.
P	780 mg.
Mg	290 mg.

* Delsterol.

in all germfree rodents, may be caused by the germfree state per se, though the possibility that one or more nutritional factors are involved is in no way excluded.

Mice. Germfree mice, of both the Swiss albino and the C3H strain, have been kept on diets L-356 and L-462. Results do not differ materially from those obtained with rats. The growth retardation observed is the same as in rats.

Rabbits.[12] Germfree rabbits have been obtained by cesarean section in the usual way. The hand-feeding period does not seem to offer unusual difficulties. The postweaning period, however, still gives unsatisfactory results and the diet is recognized as one of the primary causes of difficulty. The diet most frequently used is based on Purina Rabbit Chow,* fortified with 10 per cent casein. To two parts of this mixture, 1 part of bran plus a vitamin mix is added (TABLE 2). Sterilized, this diet gave good results in normal stock animals, but most germfree animals were lost after a few months on this diet, although some survived for nearly one year. Ceca were badly distended in most cases. Weight gain was irregular, and muscular wasting and renal cortical nephrosis were seen in many of the animals. As far as is known, however, both choline and vitamin E have been available in sufficient amounts to the animals from birth, while an acceptable balance between Ca, P, and Mg seems to exist in the diets.

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* Product of Purina Mills, Buffalo, N. Y.

Another solid diet has been tried on rabbits. This diet, developed by Bruce P. Phillips as a formula for guinea pigs, and described by him elsewhere in this monograph, consists of equal parts of Quaker Oats* and Purina Lab Chow† with some yeast and vitamins added. When used with germfree guinea pigs, results were satisfactory. With rabbits, the results were no better than those obtained with the first diet.

Guinea pigs. The work with germfree guinea pigs is discussed extensively elsewhere in these pages by Phillips. Here it suffices to mention that newborn guinea pigs can be placed immediately after birth, on the diet mentioned above for rabbits. A reduction in food supply of about 30 per cent reduced the growth of the animals but led to comparably less distended ceca. Healthy animals of ages up to 12 months have been obtained in this way (the oldest animals then became contaminated). Pregnancies did not occur, though viable sperm was present in the males.

Liquid Milk Formulas in the Preweaning Period

Rats. As pointed out by Pleasants elsewhere in this publication, the milk formulas on which the present rat and mouse colonies were originally obtained still showed signs of imperfection. To solve some of these problems, work was started to develop a milk formula better suited for the baby rat and to obtain some insight into the problem of early digestion. Most of this work was done in a non-germfree but partly shielded environment. Our best results have been obtained by using diet L-449C. This diet closely resembles the composition of natural rat milk (TABLE 3). However, the protein concentration in our milk formula could not be raised to more than 6 per cent without encountering digestive problems, as indicated by substantial amounts of undigested protein throughout the entire length of the small intestine of the rat. The reason for this is not yet clear, but certain clues may be given.

Platt[14] has pointed out that the proteolytic digestion in the stomach of the baby rat takes place by regurgitated intestinal enzymes. Using the agar-plate method of Stansly and Rainsey,[15] we found that the stomach contents of 7-day-old mother-fed baby rats showed definite proteolytic activity at *p*H about 7.5, indicating the presence of trypsin and/or chymotrypsin, but no activity at *p*H 1.6. Color reactions on sections of the filled stomach (sectioned longitudinally in half when frozen) indicated the presence of bile thoughout the stomach (nitric acid coloration) and extensive liberation of amino groups (ninhydrin coloration). The *p*H of the stomach contents centers about 5.6, which apparently makes proteolysis possible, as judged by the presence of

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* Product of Quaker Oats Company, Philadelphia, Pa.

† Product of Purina Mills, Buffalo, N. Y.

TABLE 3
MILK DIET 449C
(Rats and Mice)

Milk, homogenized	50 ml.
Cream, coffee	50 ml.
dl-Methionine	0.2 mg.
l-Tryptophan	0.06 mg.
Skim milk powder (lactose free)	4 gm.
Salts and vitamins in various solutions*	13 ml.

Composition calculated per 100 gm.

	449C	Rat milk†
Solids, total	21.8	26.9 gm.
Fat	10.0	8.9 gm.
Protein (N × 6.25)	5.5	8.7 gm.
Lactose	4.3	3.4 gm.
Ash	1.5	1.2 gm.
Ca	175.0	246 mg.
P	214.0	237 mg.
Calories	135.0	135

* For detailed composition see Pleasants elsewhere in this monograph.
† Average composition from 3 to 21 days calculated from Luckey *et al.*[13]

free NH_2 groups. Upon hand-feeding, for some unknown reason, the *p*H in the stomach is lowered and some premature secretion of pepsin sets in, as indicated by a slight proteolytic activity at *p*H 1.6. Evidently, during most of the hand-feeding period, the *p*H is not favorable for peptic or tryptic digestion (FIGURE 1). As a result, proteolysis in the stomach is slow and insufficient, as indicated by the virtual absence of ninhydrin coloration (TABLE 4). Milk protein apparently is insufficiently predigested in the stomach to allow a load of more than 6 per cent in the diet. As a result, total protein intake becomes a limiting factor. Addition of protein hydrolyzates to the diet, which has given good results to investigators at the University of Lund, in Lund, Sweden, in our hands did not produce the required results. Since in 24 hours only a limited volume can be fed, the restricted protein intake results in weight gains of about 40 per cent of the normal 1.2 gm./day. Thus far, diet L-449C has been used only to a limited extent on germfree rats and mice. However, a modification of it has been used extensively in rearing germfree rabbits.

Rabbits. In the rabbit, the digestive problems outlined above do not seem to exist to the same extent. Using formula L-449C as a basis, a higher percentage of milk protein (as high as 8 per cent) could be incorporated in the diet. Addition of more protein creates the technical problem of a diet which, following sterilization, is difficult to feed because of its texture. Also, a comparatively higher food intake than in the rat is tolerated, resulting in a better but not yet optimal gain in weight.

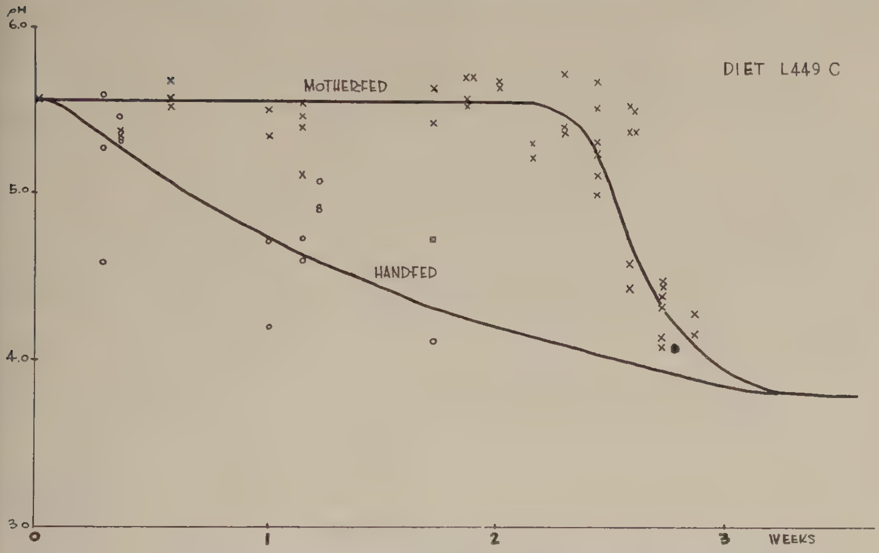

FIGURE 1. Graphic representation of the *p*H values found in the contents of the stomachs of mother-fed baby rats and baby rats hand-fed on diet L-449C after the homogenization of the total contents in 2 ml. saline.

TABLE 4
QUALITATIVE ANALYSIS OF THE STOMACH CONTENTS OF 7-DAY-OLD NORMAL STOCK RATS (LOBUND STRAIN)

	Mother-fed	Hand-fed
*p*H	5.8	4.6
Proteolytic activity at *p*H 1.6*	0	1
Proteolytic activity at *p*H 7.5*	3	4
Bile (nitric acid coloration)	+	+
Free amino groups (ninhydrin coloration)	+	−

* Arbitrary units.

Conclusion

The data presented indicate that in some instances, a workable solution has been found. In others, as in the case of the rabbit, many problems remain to be solved. However, the general problem is certainly larger than indicated here. Years ago, while visiting Lobund Institute, H. Dam from Copenhagen, Denmark, made the statement that now most nutritional work would have to be repeated, using the germfree animal.

However, in deciding how far we should go in this direction, we must weigh carefully the useful knowledge to be expected from such a program, considering the facilities and animals available for this and for other at least equally useful purposes.

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Discussion of the Paper

F. J. Orland (*University of Chicago, Chicago, Ill.*): This matter of the enlarged cecum is of considerable interest. Since Wostmann has raised the question, would he care to comment on his observations relative to monocontaminated animals? Was this a different type of enlargement from that seen in the germfree animals?

Wostmann: I do not have all the data here, but my impression is that in most cases of monocontamination the cecum showed practically the same degree of enlargement as in the germfree animal.

B. E. Gustafsson (*University of Lund, Lund, Sweden*): With reference to the papers of Wostmann and Pleasants: I noticed that there was a rather slow weight increase in the hand-fed rats and that the fat content of the milk was 9 per cent. We have found that we can increase the weaning weight of hand-fed rats to 20 gm. by increasing the fat content of the diet to about 20 per cent. With this high fat content of the milk, feeding was necessary only every fourth hour and not during the nights. I may add that we have found lysine and tryptophan to be determining factors for the numbers of animals that we could rear successfully. Finally, the amount of tocopherol acetate must be kept rather high, even in the milk formula, in order to produce the second generation of germfree rats.

STUDIES ON REARING THE GUINEA PIG GERMFREE*†

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The guinea pig was the first of the common laboratory animals to have been obtained and reared germfree. Nuttall and Thierfelder[1-3] (1895, 1896, and 1897), obtained guinea pigs by cesarean section and reared them, free of microbial agents, for a period of ten days. Although the rearing of animals germfree for such a short time would appear insignificant when compared with more recent results, these pioneering efforts were to comprise an important milestone in research on germfree life. The studies demonstrated that life without bacteria was indeed possible, and thus provided a stimulus for additional investigation.

The selection of the guinea pig as the animal of choice in the early investigations appears to have been based upon rational considerations. Investigators already knew that guinea pigs could be reared from birth on a diet of cow's milk, and they also considered the fact that the guinea pig is in a more advanced state of maturity at birth than are other common laboratory mammals. Due to the limitations of the early germfree equipment, microbial contamination of animals could be prevented for only a short period of time; otherwise, these workers might have found, as we have, that cow's milk alone is inadequate for sustaining prolonged growth of the germfree guinea pig. Although the mature status of guinea pigs at birth was taken into consideration, it was only very recently, during the investigations reported here, that this maturity was employed to full advantage in efforts to rear the animals free of microbial agents.

The methodology for rearing guinea pigs germfree has not kept pace with that for certain other animals. For example, rats and mice have been maintained germfree through more than 10 generations at the Lobund Institute. Nevertheless, some progress in the rearing of germfree guinea pigs has been realized since the time of the early experiments. Cohendy and Wollman (1914) reared guinea pigs germfree for a period of 29 days, and later (1922) these investigators reared similar animals for use in a study of cholera. Glimstedt (1932, 1936), employing facilities that were greatly superior to those of the early workers, succeeded in maintaining guinea pigs germfree for a period

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* The term "germfree" has come into accepted general usage in referring to animals reared under aseptic conditions sufficient to ensure a bacteria-free environment but not necessarily eliminating the possibility of viral or rickettsial infection. For the sake of convenience and continuity, the term germfree is used in this paper with the reservation that this connotation does not imply other than a bacteria-free history.

† The work reported in this paper was conducted at the Lobund Institute and was supported in part by Contract NR131-167 between the Office of Naval Research, Department of the Navy, Washington, D. C., and the University of Notre Dame.

of 2 months. In 1955 Miyakawa reported successful rearing of the guinea pig germfree for a period of 150 days.

Our interest in the germfree guinea pig was born of a desire to use the animals in studies of the microbial interrelationships in amoebiasis. The investigations were initiated in 1952 and have continued without interruption up to the time of this report. During the first 6 months of the investigations efforts were directed almost exclusively toward alleviating some of the problems of obtaining and maintaining the germfree animals. Since that time our efforts have been divided between a continuing study of the germfree guinea pig and the utilization of the animals in investigations of amoebiasis. Results of the latter studies have been reported in part (Phillips *et al.*, 1955, Phillips, 1957, and Phillips *et al.*, 1958). Results of efforts to rear the guinea pig germfree, together with a study of certain of their characteristics, provide the basis for this report.

Obtaining the Germfree Animals

All germfree guinea pigs were obtained by cesarean section within an operating unit of the Reyniers germfree (GF) system (Reyniers, 1956). The animals were then passed into a gf rearing unit wherein they remained for the duration of their lives. The pregnant guinea pigs were provided by the National Institutes of Health (N.I.H.) and were shipped to the Lobund Institute in the later stages of gestation. The Hartley and N.I.H. strains of guinea pigs were used in the studies, but differences were observed neither in the response of the 2 strains to the germfree state, nor in their nutritional requirements. The results obtained with the two strains will be combined.

During most of the first year we were unable to select animals at the proper stage of the gestation period for the cesarean sections. Approximately 80 per cent of the animals were used for cesarean section at an improper time, and many immature fetuses were obtained. A variety of procedures was employed in an attempt to obviate this complication. The fetuses were palpated for size and their position in the dams, and their movements were observed. In some instances the approximate breeding date of the animals was ascertained. None of these procedures, however, produced favorable results. Later, the cesarean sections were performed at a time when milk could be expressed from the teats of the dams. This procedure, although it had been employed with some success by previous investigators (Nuttall and Thierfelder, 1895), proved to be unsatisfactory in our studies. The pregnant animals sometimes delivered their young prior to the appearance of milk; in other instances, animals were observed to have milk at a time when the young were still immature.

During the second year of the investigations a method was devised that ensured the obtaining of mature young from the cesarean sections. After the procedure had been employed for almost a year we discovered that an almost identical procedure had been devised and reported by Glimstedt (1936). This technique is based upon radiological observations of the pubic bones of the pregnant animals. These observations revealed that diastasis of the epiphysis of the pubic bones occurs in pregnant guinea pigs as they approach parturition.

Two weeks prior to parturition the symphysis pubis no longer exists in its original form, and the pubic bones have separated a distance, on the average, of 6 mm. (FIGURE 1). During the ensuing period, diastasis increases at the rate of approximately 0.8 mm. per day until it reaches 17 to 18 mm. (FIGURE 2). This is followed by more rapid diastasis during the next 24 to 36 hours, at which time the bones separate until there is a distance of 21 or 22 mm. between them (FIGURE 3). It was during this period of rapid diastsais that the cesarean sections were performed with the most favorable results. We observed, however, that germfree guinea pigs derived from dams with a pubic spread of 16 mm. or more could usually be reared without complications attributable to immaturity.

Later we discontinued the radiological observations of pregnant animals. We found that the animals could be selected quite satisfactorily for the cesarean sections by feeling the position of the pubic bones with the thumb of the left hand while supporting the animal with the right hand (FIGURE 4). The pubic spread could be estimated with but little practice and the approximate parturition time determined by this procedure. It appeared unimportant whether the degree of diastasis was determined by radiological observation or by

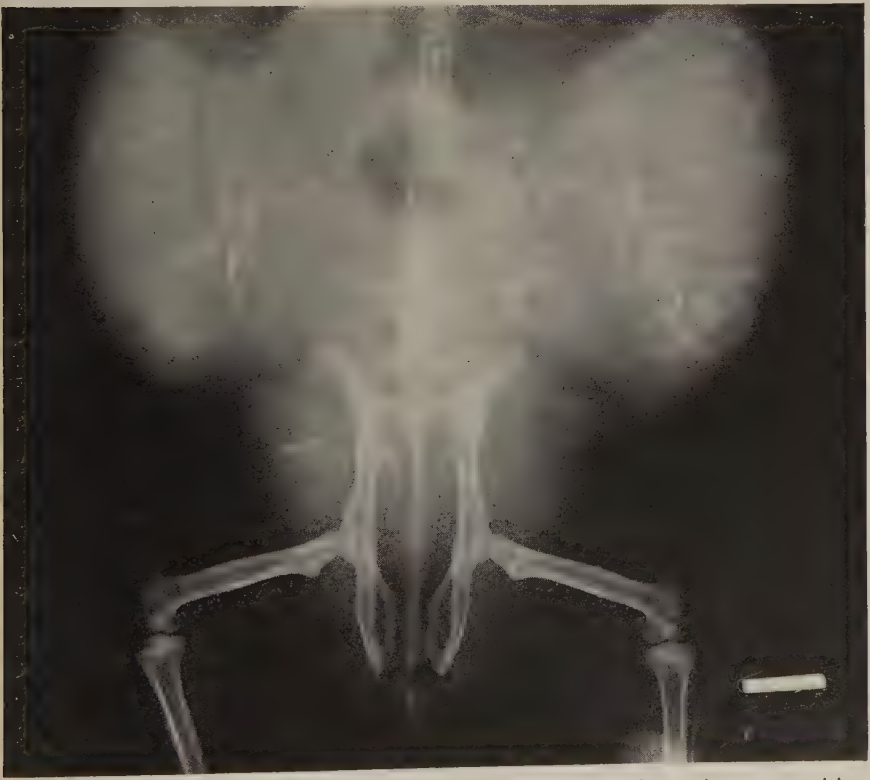

FIGURE 1. Radiograph of the pubis of a gravid guinea pig 14 days prior to parturition. Diastasis of the pubic bones is 6 mm.

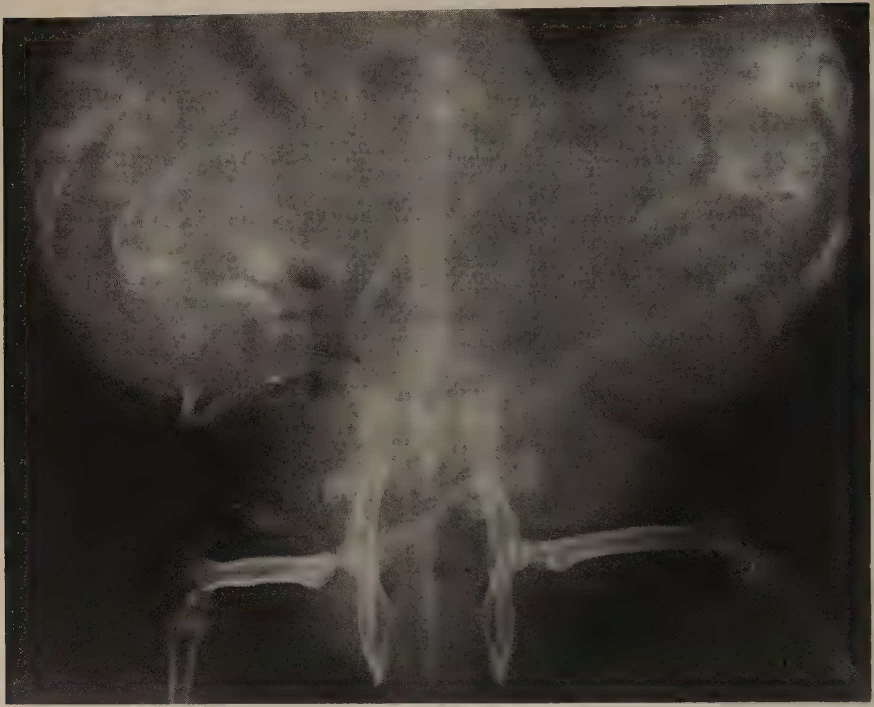

FIGURE 2. Radiograph of the pubis of a gravid guinea pig 24 hours prior to parturition. Diastasis of the pubic bones is 17 mm.

palpating the pubic region of the dams by the method we eventually adopted. Radiological observation permitted a predetermination of the number of fetuses present, and larger offspring were usualy obtained if dams with small litters were selected for the cesareans. However, smaller animals obtained from litters up to six in number appeared to be as easily reared germfree as were the larger offspring.

Housing the Germfree Animals

Several methods for housing guinea pigs within the germfree tanks were evaluated. In some experiments the animals were permitted to run together within a single large enclosure. In other instances the animals were placed individually into 7- by 9-inch metal cages with wire-mesh bottoms. Both methods had advantages and disadvantages of some significance. Animals that were permitted to run together exhibited what may be called, for convenience, a suckling instinct: they attempted to nurse each others' anal regions. Although this tended to stimulate the passage of feces and urine and thus eliminated the potential need for artificial stimulation of the animals for the first 3 to 5 days of life, more prolonged activities of this nature were often accompanied by undesirable consequences. More animals developed anal prolapse when housed together. Animals feeding from the same dish tended to

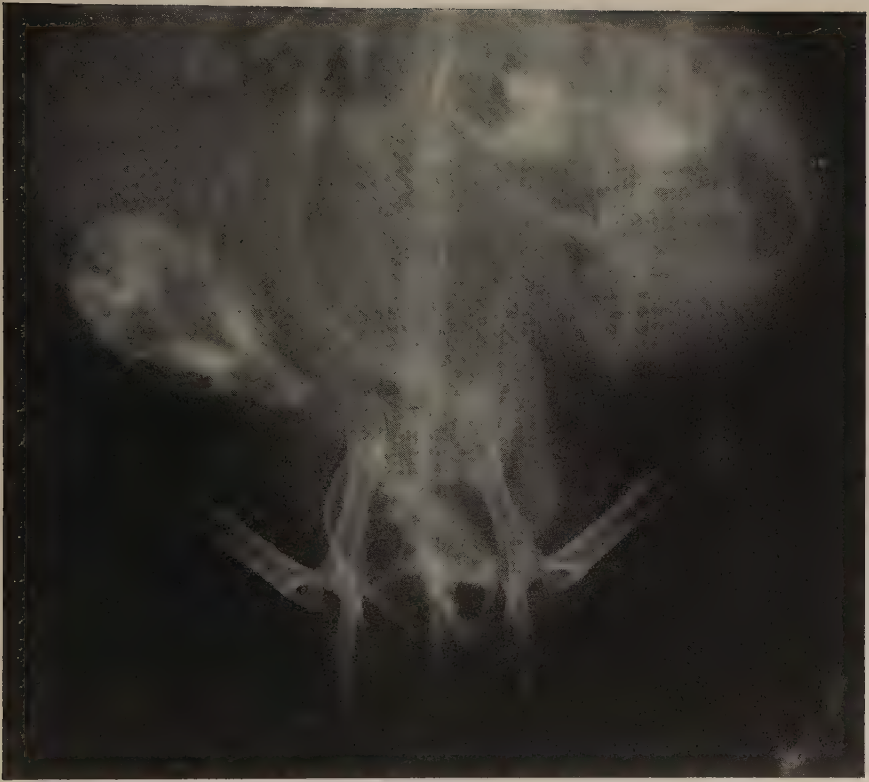

FIGURE 3. Radiograph of the pubis of a gravid guinea pig 25 minutes prior to parturition. Diastasis of the pubic bones is 22 mm.

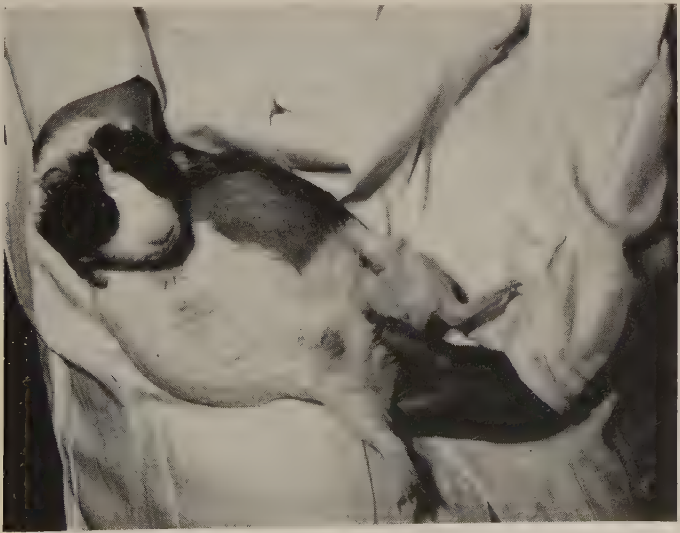

FIGURE 4. The degree of diastasis of the pubic bones in the gravid guinea pig may be estimated by palpation of the pubis, as shown.

compete for the diet at the time of each feeding, even though the quantity of diet was greater than they could consume collectively. As a result, the feeding period represented an exhausting experience for many of the animals and, at its conclusion, the fur of most of them was usually covered with the diet. An occasional animal developed an appetite for the fur of others, and consumed large amounts of it within a few days. Although the hair-eaters appeared to suffer no deleterious effects from the ingestion of large quantities of fur, their victims would often stop eating, become moribund, and die for no other reason that was grossly apparent. Attempts to discourage the suckling instinct and the hair-eating habit of the animals by dipping the other guinea pigs in various unpalatable solutions were without success.

Although the isolation of the animals into individual cages from birth obviated the aforementioned complications, it had inherent though not too serious disadvantages. Animals confined to the small cages were slow to develop an appetite. Their ability to eliminate waste materials from the intestine seemed to be inferior to that of some animals that were allowed more exercise in the large runs and, as a result, there was more cecal distension. Eventually, the following combination of housing procedures was adopted as the method of choice.

Following cesarean sections, the animals in groups of 3 were placed in the small cages until they reached an age of 5 days. They were then isolated into individual cages until they reached an age of approximately 60 days. At this time the suckling instinct had usually disappeared, and the hair-eating tendency had not developed. The animals were then transferred to a larger pen and allowed to run together for the remainder of their lives. Rarely, a hair-eater developed at this later stage, in which case it was either segregated or destroyed before extensive damage was inflicted upon the others.

Wood shavings, muslin towels, and paper were employed as bedding for animals of the various experiments. The bedding of choice appeared to depend upon the age of the animals. Wood shavings showed good absorptive qualities and provided a bedding that remained drier for longer periods of time than the other materials. The germfree guinea pigs ingested the shavings in large quantities and experienced difficulty in passing this material through their digestive tracts, this was especially true of the young animals. The shavings often became impacted in the ceca of the animals, thus retarding the passage of waste materials into the large intestine. Consequently it was found more desirable either to bed the young animals on muslin towels, which they consumed in lesser quantity, or to employ no bedding materials until they reached an age of 8 to 10 weeks. The older guinea pigs, however, appeared more capable of coping with ingested wood shavings although, even in these animals, the ingestion of such material appeared to retard the passage of feces. Germfree guinea pigs fed our most successful dietary regimen passed fecal pellets of soft consistency. When these animals were placed on a bedding of wood shavings, however, they passed fecal pellets of a consistency similar to that of conventional guinea pigs on commercial rations.

Temperatures of 36 to 37° C. were maintained within the germfree tanks during the early experiments. Later, when more mature young were obtained from the cesarean sections, lower temperatures were employed with good results. In recent experiments, a temperature of 32 to 33° C. was maintained until the animals reached an age of 7 days. This was followed by a 3-week period during which the temperature was maintained at approximately 31° C. Thereafter, an effort was made to maintain the temperature at 30° C. until the animals reached an age of 6 months. Older animals were maintained at a temperature of approximately 28° C. All of the aforementioned temperatures were maintained at a central point in the housing unit approximately 12 inches above the floor of the cages. However, since heat was supplied to certain portions of the walls of the tanks with infrared lamps, there was some variation of temperature in different areas of the housing units. The temperature of air exhausted from the tanks was usually 2° lower than that at the aforementioned central point.

Determining the Microbiological Status of the Germfree Animals

The microbiological status of the animals was determined at intervals of not more than one week. Samples of bedding, diet, water, freshly passed feces, and other materials within the germfree tanks were removed and examined for contaminants. These samples were employed as inocula for fluid thioglycollate and blood-brain-heart-agar media. The media, in triplicate sets, were incubated at 25°, 37°, and 55° C. for a minimum period of 2 weeks. These were examined periodically for the presence of bacteria, yeasts, and molds.

Experimental Diets, Vitamin Supplements, and Feeding Procedures

The diets employed in the studies were of 3 types: liquid, dry, and semisolid. The diets were fed to a minimum of 12 litters of germfree guinea pigs. Diets were evaluated on the basis of sustaining life and promoting growth in the animals. Only the diets palatable to the animals are described here. The formulas for the diets are presented in TABLES 1, 2, and 3. The liquid diets in glass ampules, the dry diets in cloth bags, and the semisolid diets in refrigerator-type ice-cube trays were autoclaved into the germfree tanks at 250° F. for 20 min. The pans of semisolid diets were covered with aluminum foil while hot to retard evaporation during storage within the germfree tanks. During the early studies liquid diets were hand-fed to the animals with an ordinary medicine dropper equipped with a rubber bulb. In most instances restricted quantities of the diets were administered to the animals at intervals of 1 or 2 hours on a 24-hour schedule each day. In several experiments, however, the animals were given all they cared to consume at each feeding. Later the liquid diets, in bottles equipped with a self-feeding dropper, were attached to the cages, and the animals were permitted to feed *ad libitum*.

Some of the animals were transferred from liquid diets to dry diets upon reaching an age of 3 to 6 weeks. This transfer was usually accomplished over a period of 1 week. During this time the dry diets were made available to the animals *ad libitum*, and there was a gradual diminution in the number of hand

Table 1

Formulas for Diets Employed in Studies on the Rearing of Germfree Guinea Pigs

Number and Type of Diet

Components (gm.)	L-228 L*	L-253 L	L-259 L	L-301 L	L-325 L	L-229 D†	L-365 D	L-385 D	L-389 D
Liver, powdered						30	30		
Yeast, powdered						20	20		
Casein, Labco						300	300		
Casein, #24‡				8					
Casein, Sheffield		5						300	300
Cornstarch						290	290	300	300
Pablum				1					
Cream, 20 per cent			79	75	79				
Coconut oil		2.4							
Corn oil	100					50	50	80	80
Sucrose	46		20		4				80
Lactose								80	
Lactalbumin		2							
Gum arabic	15					150	100		
Lard		5.6							
Whey salts		89							
Salt mix II§	4			0.5		60	60	60	60
Lecithin		0.5							
Alphacel						70	120	120	120
Cellophane								40	40
Potassium acetate	2.5					20	20	20	20
Magnesium oxide	0.5					4	4	2	2
Menadione	0.005								
Delsterol (D_3)	0.0002								
B-carotene	0.03								
Ascorbic acid	1	0.5	1	0.5	0.2	5	5	10	10
Mixed tocopherols	0.03								
Vi-syneral			0.3	0.2	0.3				
B complex-1A	0.8								
B complex-47		5							
B complex-67						10	10		
B complex-88								5	5
Ladek-8		1							
Ladek-26						5	5		
Ladek-29								10	10
Water				15	17				

* Liquid.

† Dry.

‡ Prepared from skim milk at the Lobund Institute.

§ Formula in grams: $CaCO_3$, 1200; $CaHPO_4$, 900; Na_2HPO_4, 800; NaCl, 200; KI, 3; $MgSO_4 \cdot 7H_2O$, 171; $MnSO_4 \cdot 4H_2O$, 38; Fe $(C_6H_5O_7)_2$, 300; $CuSO_4$, 11; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnSO_4$, 2.5; $Na_2B_4O_7 \cdot 10H_2O$, 2; $AlK(SO_4)_2 \cdot 12H_2O$, 3.

feedings and the quantity of liquid diet administered. Subsequent studies revealed that it was unnecessary to hand-feed the animals during the period of their infancy. Instead of administering liquid diets by dropper, containers of dry or semisolid diets and water were placed in the cages with the animals within a few hours following their delivery. The results thus obtained were so favorable to maintenance of the animals that the hand-feeding and use of liquid diets were discontinued.

TABLE 2

FORMULAS FOR DIETS EMPLOYED IN STUDIES ON THE REARING OF GERMFREE GUINEA PIGS

Number and Type of Diet

Components (gm.)	L-339 S*	L-342 S	L-455 S	L-463 S	L-393 D†	L-GP-6 L‡
Trypticase (BBL)						2
Purina Lab Chow	100	150			50	
Quaker Oats	50	75	40	100	25	
Casec	10	19				
Celluflour	8					
Cellophane	12					
Cerophyl			5		7.5	
Alfalfa leaf meal			20			
Soya bean meal			5			
Rice flour			5			
Wheat germ				100		
Milk, homogenized		62				60
Cream, 20%		31				37
Cottonseed oil			10			
Sucrose	20				1	
Dextrose			8	10	2	
Saliva, human	4					
Lactalbumin	60	1				
Salt mix II §		0.5				
Alphacel					10	
Sodium chloride			4	5	0.4	
Potassium chloride			1			0.5
Calcium carbonate			2		1	
B-carotene					0.2	
Ascorbic acid					3	0.5
Riboflavin					0.04	
Thiamine HCl					0.04	0.1
B complex-67	3					
Water	500	780	1000	1000		

* Solid.

† Dry.

‡ Semisolid.

§ Formula in grams: $CaCO_3$, 1200; $CaHPO_4$, 220; K_2HPO_4, 900; Na_2HPO_4, 800; NaCl 200; KI, 3; $MgSO_4 \cdot 7H_2O$, 171; $MnSO_4 \cdot 4H_2O$, 38; Fe $(C_6H_5O_7)_2$, 300; $CuSO_4$, 11; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnSO_4$, 2.5; $Na_2B_4O_7 \cdot 10H_2O$, 2; AlK $(SO_4)_2 \cdot 12H_2O$, 3.

Semisolid diets have been employed in our studies for the past 3½ years. Although these diets have been used by a variety of procedures, in diverse quantities and consistencies, the following method was adopted as best for their administration. The semisolid diet is given to the germfree animals within a few hours following their cesarean birth. During the first week the diet, diluted to a fluid consistency with water, is placed in shallow dishes and fed to the animals *ad libitum*. During the second and third weeks the quantity of water added to the diet is reduced gradually to approximately 30 per cent by volume or in sufficient quantity to render it the consistency of a soft paste. The diet is administered in this consistency thereafter. Feeding *ad libitum* is discontinued at the end of the first week. Thereafter, the animals are fed twice daily, at an interval of 7 to 8 hours. During the second week the animals are allowed to eat as much of the diet as they care to consume within a period

TABLE 3

FORMULAS FOR DIETS EMPLOYED IN STUDIES ON THE REARING OF GERMFREE GUINEA PIGS

	Number and Type of Diet								
Components (gm)	L-326 S*	L-350 S	L-445 S	L-364 D†	L-370 D	L-378 D	L-382 D	L-412 D	L-428 D
Purina Lab Chow	200	90	100	200	400	400	56	200	
Quaker Oats	100	45	100	100	200	200	28	100	30
Cellophane		15							
Cerophyl						12	2	6	30
Dextrose			10		12	24	3	12	56
Sodium chloride			5		12	6	1		2
Calcium carbonate						9	1	3	30
Ascorbic acid				30	30	30	4		
Water	1000	500	1000						
Alphacel							5	15	
Calcium chloride								3	2
Mixed tocopherols								2	5
Alfalfa leaf meal									75
Corn oil									30
Rice flour									30
Soya bean meal									15
Whole dry yeast			15						

* Semisolid.
† Dry.

of 1 hour at the morning feeding and are given approximately 50 per cent more at the afternoon feeding. By the end of the second week the average germfree guinea pig consumes approximately 40 to 45 gm. of the diluted diet (10 to 12 gm. dry weight) daily. The animals are restricted to this quantity of food until they reach an age of 6 weeks. Thereafter, the amount fed is increased, periodically, until the animals are consuming approximately 120 gm./day (approximately 35 gm. dry weight) upon reaching adult size at the age of 6 months. There is no further increase in the amount of food given to the animals, regardless of age. Autoclaved tap water, in round porcelain jars, is made available to the animals *ad libitum* during their entire lives.

All of the aforementioned diets either contained or were supplemented by certain vitamin preparations. The formula for these preparations are presented in TABLES 4 and 5. All animals on liquid or dry diets that did not contain ascorbic acid were given 1.0 ml. of ascorbic acid preparation VC-1 (TABLE 5) orally, each day. All semisolid diets were supplemented with ascorbic acid preparation VC-2 (TABLE 5). This was mixed with the diet at one of the feedings each day at a minimum rate of 1.0 ml. per animal. The vitamin B preparations 1A, 47, 67, and 88 (TABLE 4), and the Ladek preparations 8, 26, and 29 (TABLE 5), were added to the liquid and dry diets as shown in TABLE 1. Vitamin B preparations GP-1,-2,-3, and -4, (TABLE 4) were employed individually and administered orally, by dropper, to certain animals reared on dry diets L-364, -370, -378, -382, -412, and -428 (TABLE 3). The animals received 1.0 ml. of the preparations twice daily. Wheat germ oil was employed in only one experiment; this involved animals maintained on the L-445 regimen.

TABLE 4

FORMULAS FOR B VITAMIN PREPRATIONS EMPLOYED EITHER IN VARIOUS GUINEA PIG DIETS OR AS SUPPLEMENTS TO THEM

Components (gm.)	1A	47	67	88	GP-1	GP-2	GP-3	GP-4
Yeast, powdered					1.5	0.25	0.25	
Liver, powdered					1.5	0.25	0.25	
Calcium pantothenate	0.06	0.002	0.01	0.008	0.1	0.05	0.25	0.15
Choline chloride				0.2	0.3	0.25	0.05	0.005
Methionine					0.3	0.25	0.05	0.05
i-Inositol	4	0.005	0.1	0.2	0.3	0.25	0.05	5
Folic acid		0.002	0.001	0.002	0.03	0.005	0.002	0.04
Vitamin B_{12}		0.01 mg.	0.01 mg.	0.05 mg.	0.1	0.001	0.002	0.005
Pyridoxine HCl	0.02	0.001	0.003	0.002	0.015	0.01	0.05	0.15
Potassium iodide					0.02			
Cobalt chloride					0.005	0.001	0.003	0.003
Magnesium sulfate					0.1	0.03	0.03	0.03
Copper sulfate					0.05	0.02	0.05	0.025
Potassium chloride					0.8	0.25	0.25	0.25
Niacinamide		0.002	0.01		0.3	0.125	0.5	
Riboflavin	0.028	0.002	0.003	0.003	0.2	0.075	0.5	0.15
Thiamine HCl	0.02	0.003	0.01	0.01	0.5	0.25	1	0.1
Calcium phosphate					0.5	0.5	2	1
Manganese sulfate					0.1			0.05
Ascorbic acid					3			2
Ferric pyrophosphate							0.5	0.25
p-Aminobenzoic acid	0.2		0.005					0.025
Nicotinic acid	0.2			0.01				0.005
Biotin		0.01 mg.	0.1 mg.	0.1 mg.				0.004
Dextrose					3	15		
Cornstarch			to 1	to 0.5				
Dist. water		to 5			to 100	to 500	to 500	to 500

TABLE 5

FORMULAS FOR LADEK* AND ASCORBIC ACID PREPARATIONS EMPLOYED EITHER IN THE VARIOUS GUINEA PIG DIETS OR AS SUPPLEMENTS TO THEM

Vitamin (gm.)	Ladek 8	Ladek 26	Ladek 29	VC-1	VC-2
A, concentrated	0.025	0.03	0.05		
D, Delsterol	0.005	0.005	0.01		
E, mixed tocopherols	0.150	0.5	0.3		
K, menadione	0.002	0.02	0.01		
Ascorbic acid				1	1
Thiamine					0.05
Corn oil to	10	10	10		
Water				100	100
Dextrose				1	

* Vitamins A, D, E, and K.

A commercial preparation, Ganatrex,* was used as a supplement to diet L-445 in a series of 7 experiments. The wheat germ oil and the Ganatrex were mixed with the diet at one of the daily feedings at the rate of 1.0 ml. each per animal.

* Ganatrex is the proprietary name of a metabolic supplement produced by The Wm. S. Merrell Co., Cincinnati, Ohio.

All vitamin supplements were autoclaved into the germfree tanks at 250° F. for 20 min. The wheat germ oil, in glass ampules, was also presterilized with dry heat. Chemical assay of the VC-2 vitamin preparation before and after autoclaving showed less than 25 per cent loss in potency of both the ascorbic acid and thiamine HCl as a result of the sterilization treatment. Other vitamin preparations were not assayed.

Results Obtained with the Various Dietary Regimens

Detailed results of each of the 135 experiments are too extensive for presentation here. Cesarean sections were performed on 362 dams, and more than 1000 germfree young were obtained. During the early period of the investigations germfree guinea pigs could be reared only for periods of 1 to 3 weeks. This was due, undoubtedly, to a combination of factors. Many of the animals were obtained in a somewhat premature condition, the diets were inadequate, and methods for feeding and handling the animals had not been perfected. Eventually, however, satisfactory procedures were developed for maintaining the animals through the normal suckling period. A fortified cow's milk diet, L-GP6 (TABLE 2), provided a highly palatable regimen that sustained some growth of the animals for a period of approximately 3 weeks. During the fourth week, however, the general health of the animals became impaired, and it was not possible to maintain them in a state of good health thereafter. A few of the animals were reared to the age of about 10 weeks by continuing the hand-feeding beyond the natural weaning age. The growth rate after 3 weeks, however, was unsatisfactory and the animals eventually succumbed, to what was presumed to be inadequate nutrition.

Animals fed the same milk diet *ad libitum* during the natural postweaning stage developed greatly distended ceca. This progressed until the cecum became sufficiently large to exert pressure upon the folds of the lower colon. The animals then succumbed, seemingly as a result of their inability to eliminate waste materials from their digestive tracts. The results obtained by using the other liquid diets listed in TABLE 1 (L-228, -253, -259, -301, and -325) were less satisfactory; the animals survived for shorter periods of time on these preparations. Similar results were obtained when animals were fed either buttermilk, or a preparation of one part guinea pig milk to one part water. Suffice it to say that, of all the liquid diets employed, the fortified cow's milk diet gave the most favorable results.

Germfree guinea pigs weaned from the liquid diets onto the dry diets —L-229, -365, -385, -389 (TABLE 1), -393 (TABLE 2), -364, -370, -378, -382, -412, and -428 (TABLE 3)—lost an appreciable amount of their body weight during the weaning process. This was believed to be due, to a large extent, to the difficulty of adapting the animals to the new diets. The animals, although obviously hungry, were quite reluctant to consume food with which they had not become familiar during the period of their infancy. Most of the animals became accustomed, eventually, to rely on such nutriment for their maintenance, and they consumed the food on their diets in considerable quantity. The results, however, left much to be desired. Of the various dry diets employed, L-412 gave the best results. Approximately 50 per cent of the animals on this diet survived for periods of 2 to 3 months. A few of the animals were maintained for approximately an additional month. None of the animals, however, was of more than fair quality. The ceca were seriously distended. The weight gain, although fair, was misleading; much of this was attributable to a gradual increase in the quantity of cecal content. Other characteristics included a greatly distended gall bladder, a conspicuous absence of fat deposits, and poor skeletal and muscular development.

Later it was observed that animals of similar quality could be reared for comparable periods of time by feeding the diet L-412 from the time of birth. It became evident also in later experiments that restricting the dietary intake of the animals was desirable. Animals on the dry diet *ad libitum* for approximately 12 hours and starved for the remaining 12 hours each day developed slightly less cecal distension; they exhibited an increased vitality; and the growth was not impaired even though their food intake was reduced by approximately 35 per cent. During this phase of the studies several autoclaved commercial dry diets were also evaluated. However, none of the commercial diets *per se* proved to be as satisfactory as diet L-412.

During the latter part of the period wherein the diet L-412 was the regimen of choice, various canned vegetables were employed either as supplements to, or as the main component of, the dietary regimen. This was primarily an attempt to provide germfree animals with the grass-juice factor to which frequent reference is made in the literature on the nutrition of the guinea pig. Canned kale, spinach, peas, mustard greens, lima beans, bean sprouts, asparagus, turnip greens, and mixed vegetables were employed. In other experiments, the infant foods, strained peas and spinach, were fed. Although the animals consumed these vegetables in considerable quantity, there was no indication that any of the preparations contributed anything toward satisfying the nutritional requirements of the germfree guinea pig. It appeared either that a grass-juice factor was not adequately present in these sterilized preparations or that it was not required by the germfree animals in quantities greater than were available in the diet L-412.

The first of the semisolid diets (L-326, -350, -445, shown in TABLE 3 and L-339, -342, -455, and -463, shown in TABLE 2) employed in the studies was L-326. This was supplemented with ascorbic acid preparation VC-2 (TABLE 5). The inadequacy of this regimen was demonstrated in a series of experiments wherein it failed to sustain life in the germfree guinea pigs for more than 20 to 40 days. This diet, however, had been employed with more success by Phillips and Bartgis (1954) in their studies with conventional guinea pigs. Furthermore, Pleasants (personal communication) reared conventional guinea pigs, of the same strain employed in the germfree studies, through 4 generations on the L-326 diet sterilized by procedures comparable to those employed in the germfree rearing units. Although these animals were of poor quality and their mortality rate was high, the maintenance of some of the conventional animals on an autoclaved diet that had proved inadequate for sustaining life in the germfree guinea pig provided some most helpful implications. The data suggested a need for considering the germfree guinea pig as a distinct entity in any attempt to satisfy the nutritional requirements of this species. It appeared either that the capacity of the germfree animal for effective utilization of nutritive materials was not comparable to that of the conventional guinea pig or that the nutritional requirements of the germfree and conventional guinea pig were dissimilar.

Continuing studies with semisolid diets were accompanied by considerable progress. Several of the diets produced results more favorable than those obtained with the L-326 regimen. Furthermore, the use of semisolid as opposed to the dry diets seemed to be advantageous. For example, in a related series of experiments, the water component of one of the most successful semisolid diets was withheld, and only the dry components were autoclaved into the tanks and fed to the germfree animals. In these instances the germfree animals consumed the diet in seemingly adequate quantities, but they grew at a slower rate and survived for an appreciably shorter time than animals that received the diet autoclaved with the water component. Although no attempt was made to evaluate the effects of sterilization on nutritive substances in the semisolid and dry diets, it would seem that deleterious effects should be greater on the former. Apparently, however, some reaction must occur during sterilization of the semisolid diet that renders its nutritive materials more readily available or digestible to the germfree guinea pig.

Of the various semisolid diets employed, L-455 (TABLE 2) and L-445 (TABLE 3) provided the most favorable results. Both regimens proved satisfactory for maintenance of germfree guinea pigs during the normal preweaning period and for promoting growth of the animals beyond this stage. Diet L-455 was employed in only a limited number of experiments involving 22 animals, but it appeared to be highly palatable and promoted satisfactory growth of germfree guinea pigs. The longest experiment employing the L-455 regimen was 92 days. The experiment began with 6 animals, 5 of which survived for its duration. The weights of the animals at 92 days ranged from 342 to 411 gm. The animals were alert, very active, and of good appearance except for the fact that their fur seemed more oily than that of conventional guinea pigs in good condition. Post-mortem examination revealed good development of the skeleton and musculature. Fat deposits were present in normal quantity. The viscera, with exception of the ceca, were normal. The ceca, however, were considerably distended.

Diet L-445, supplemented with vitamin preparation VC-2, is the regimen of our choice for rearing the guinea pig germfree. It has been employed in the maintenance of all guinea pigs used in the amoebiasis studies for the past 3½ years, and to rear guinea pigs to adult size. The animals consume very little of the food comprising this diet for the first 24 to 36 hours of their lives. During this period there is an appreciable loss of body weight. This weight loss, however, is not considered important, and is believed due, possibly, to dehydration, which subsides prior to approaching a serious level. The growth response of young germfree guinea pigs to the L-445 regimen is shown in TABLE 6. These data show an approximate 14 per cent loss in the average body weight

TABLE 6

WEIGHTS (IN GRAMS) OF 10 GERMFREE GUINEA PIGS MAINTAINED ON DIET L-445 SUPPLEMENTED WITH ASCORBIC ACID PREPARATION VC-2

Age in days

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	20	25	30
103	87	88	87	88	91	91	95	91	92	92	98	105	109	118	123	144	159
91	81	81	80	83	87	87	94	95	96	97	98	104	116	129	150	168	180
93	81	80	80	84	86	88	92	95	96	101	100	109	116	118	138	158	162
89	80	80	77	80	85	87	88	90	93	95	97	96	96	105	124	139	154
75	68	67	67	69	72	73	79	75	70	74	76	81	80	88	100	116	123
105	92	91	89	90	90	92	90	90	93	92	94	96	97	106	110	123	134
133	121	116	114	116	122	122	130	133	141	144	150	151	156	163	184	203	203
87	75	74	71	73	78	80	85	92	97	101	104	107	106	111	135	150	167
65	60	61	60	62	68	73	77	84	88	93	93	99	105	115	132	Sacrificed (anal prolapse)	
92	83	83	79	80	80	83	83	85	88	87	91	91	93	101	Died		
Av. 93	83	82	80	82	86	88	91	93	95	98	100	104	107	115	133	150	160

of 10 animals during the first 3 days of life. The average weight of the group at 8 days corresponded to the weight at birth. Although the growth response varied somewhat among individuals, all animals began to gain weight on the fourth day. During the ensuing 27 days the average weight gain of the group was 80 gm., or an average gain per day of approximately 3 gm. The 20 per cent mortality rate in the animals is not unusual; it is due in some instances to the fact that the animals are of only fair quality at birth.

Some comparative results, not presented in the table, showed that conventional guinea pigs removed from their mothers at birth could not be reared on the quantities of the L-445 regimen that were given the germfree animals. Conventional guinea pigs fed 75 to 100 per cent more of the diet than the germfree ones, however, survived and showed growth for 30 days that was very similar to that of the germfree animals. When nonsuckling conventional guinea pigs were fed nonsterilized rabbit pellets and cabbage from the time of their birth, they showed very little growth for 12 to 14 days. Growth thereafter, however, was at a rate that exceeded considerably that of either germfree or conventional animals maintained on restricted quantities of the sterilized L-445 regimen. The mortality rate in conventional guinea pigs removed from their mothers at birth was very high, being from 40 to 50 per cent regardless of the dietary regimen employed.

Data relative to the weights of older germfree guinea pigs, when sacrificed following their maintenance on dietary regimen L-445, are presented in TABLE 7. These data show that the weights of animals at the given ages varied considerably. Furthermore, although not shown in the table, this could not be attributed to any appreciable difference in the weights or quality of the animals at birth. This variability, which was considerably greater than that observed in our conventional guinea pigs, was noted in all long-term experiments. Al-

TABLE 7

WEIGHTS OF MALE AND FEMALE GERMFREE GUINEA PIGS OF VARIOUS AGES WHEN SACRIFIED FOLLOWING THEIR MAINTENANCE ON DIETARY REGIMEN L-445 FROM BIRTH

Male			Female		
Animal	Age (days)	Weight (gm.)	Animal	Age (days)	Weight (gm.)
1	46	220	1	46	172
2	57	280	2	53	206
3	57	284	3	108	285
4	62	375	4	122	320
5	86	288	5	134	510
6	148	610	6	159	342
7	159	411	7	277	486
8	183	600	8	277	510
9	339	646	9	311	615
10	400	703	10	372	612

though all the animals were maintained under similar conditions and fed similar quantities of a common dietary regimen, certain individuals appeared to become more readily adapted to life in the germfree environment and grew at a faster rate than did certain others. Although the data of TABLE 7 suggest that the growth of male animals may exceed that of females, we are not sure that this is always or even usually true. The weights of some of the adult animals, 8 months or more of age, were nearly comparable to those of conventional guinea pigs maintained on commercial diets in our laboratory.

The administration of B complex preparations GP-1, -2, -3, and -4 (TABLE 4) to germfree guinea pigs maintained on the dry diets provided no detectable beneficial results. The growth rate and survival time of the animals were not altered by any of these preparations. The autoclaved wheat germ oil, used in a single experiment, proved to be unpalatable to the germfree guinea pigs maintained on dietary regimen L-445. The results obtained, when dietary regimen L-445 was supplemented with Ganatrex, are difficult to interpret with respect to the effectiveness of this preparation. Although the growth response of the germfree guinea pigs was not altered appreciably by this preparation, there were some indications that it may be beneficial to the animals. The mortality rate in young germfree guinea pigs was negligible in 7 experiments in which the preparation was employed. There was a suggestion that the preparation or some of its ingredients may have improved the intestinal tonus of the germfree animals. Some animals of very poor quality at cesarean birth were successfully maintained on dietary regimen L-445 plus the preparation. Furthermore, of the numerous vitamin complexes employed during the course of the studies, Ganatrex showed a higher degree of palatability to the germfree guinea pigs than any of the others. As a result of these observations, Ganatrex is being employed, experimentally, as a supplement to the L-445 regimen in all our present studies. Admittedly, however, factual evidence of its value to the animals remains to be provided.

On the other hand, the L-445 diet requires supplementation with ascorbic acid. This has been confirmed in several experiments. Germfree and con-

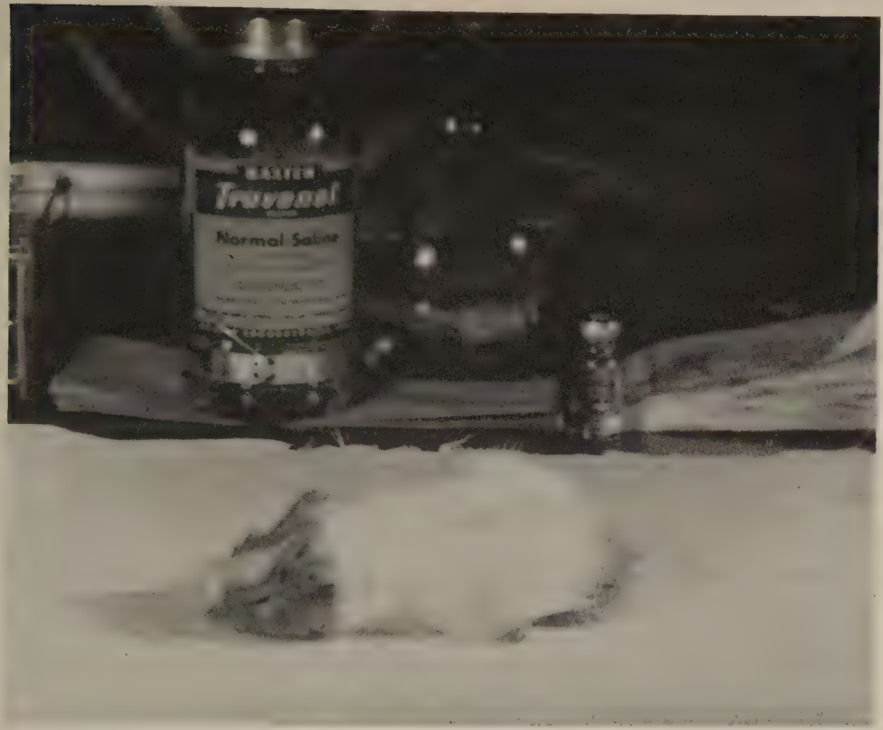

FIGURE 5. Germfree guinea pig photographed immediately following its removal from the germfree tank. This animal had been maintained for 36 days from birth on diet L-445 without an ascorbic acid supplement.

ventional guinea pigs on this diet without the VC-2 supplement or some other source of ascorbic acid develop scurvy. In a recent experiment 8 germfree guinea pigs were separated into 2 groups immediately following cesarean birth. Four animals (group A) were placed upon the L-445 diet supplemented with the usual ascorbic acid preparation VC-2 and Ganatrex. The other 4 animals (group B) received the same rations and supplement, except that ascorbic acid was omitted from preparation VC-2. The animals of group A showed satisfactory growth for a period of 90 days, the duration of the experiment. The animals of group B showed comparable growth for 26 days, but began losing weight thereafter. By day 33 all animals of group B showed an appreciable loss in body weight. Scorbutic symptoms were noted in these animals on day 28; the animals were less active and could walk only with difficulty. By day 35 all 4 animals had assumed the scurvy position, their knee joints and feet were tender and swollen, and they were unable to stand. Respiratory difficulties were pronounced.

Two of the scorbutic animals were sacrificed and examined on days 36 and 38, respectively. A photograph of the animal sacrificed on day 36 is shown in FIGURE 5. Post-mortem examination showed fairly typical lesions of scurvy in both macroscopic and microscopic observations. The other 2 scorbutic animals were given vitamin preparation VC-2 as a supplement to the diet on day 39 and thereafter. In 48 hours the animals were more alert and respiration was improved; at 72 hours the animals began to move about in their cages; and at 144 hours the animals were on their feet and the disabling symptoms of scurvy had disappeared. Comparative studies with conventional guinea pigs of similar ages and fed similar rations revealed that these animals developed scorbutic symptoms at approximately the same time as the germfree animals. The most conspicuous difference noted in the conventional guinea pigs was in the amount of time required to alleviate the scorbutic symptoms. Recovery from the symptoms of scurvy required approximately 3 times longer in the conventional guinea pigs than in the 2 germfree guinea pigs that received the same vitamin therapy.

The amount of ascorbic acid given germfree guinea pigs maintained on the L-445 regimen is several times that required to prevent scurvy. In some experiments the animals received only one third of this quantity of the vitamin and remained free from scorbutic symptoms. On the other hand, the germfree guinea pig has shown a high degree of tolerance for excessive quantities of the vitamin. Some animals were given as high as 10 times our recommended dosage of ascorbic acid without any alteration of results. The dosage recommended herein was designed to provide a margin of safety either if preparations of lower potency should be employed inadvertently, or if such preparations should be reduced in potency by excessive sterilization intervals or temperatures. The thiamine HCl component of the VC-2 vitamin preparation did not appear to be essential for the maintenance of the germfree guinea pig on diet L-445. However, this vitamin is included in the regimen because it has appeared to improve the quality of the animals. Although precise information cannot be provided as to its effects on the animals, it seems to improve the tonus of the intestines.

Certain Anatomical Characteristics of the Adult Germfree Guinea Pig

Germfree guinea pigs have been reared to adult size on the L-445 dietary regimen in several experiments. The experiment of longest duration began with 5 animals, 2 males, and 3 females, each of which was derived from a different litter. One of the males died at the age of 5 months with a perforated stomach after swallowing glass from a broken ampule. The other 4 animals were maintained germfree to the ages of 337, 337, 378, and 406 days, respectively, at which time they became contaminated, accidentally, with *Micrococcus*. In physical size and appearance the animals compared favorably with adult conventional guinea pigs of good quality. A photograph of the animals when the oldest was 1 year of age is shown in FIGURE 6. One of the animals was also photographed upon removal from the germfree tank just prior to sacrifice (FIGURE 7).

The animals were alert and active throughout their lives. On external examination the guinea pigs appeared well developed and in good state of nutrition. The skin and fur were exceptionally clean and of normal texture.

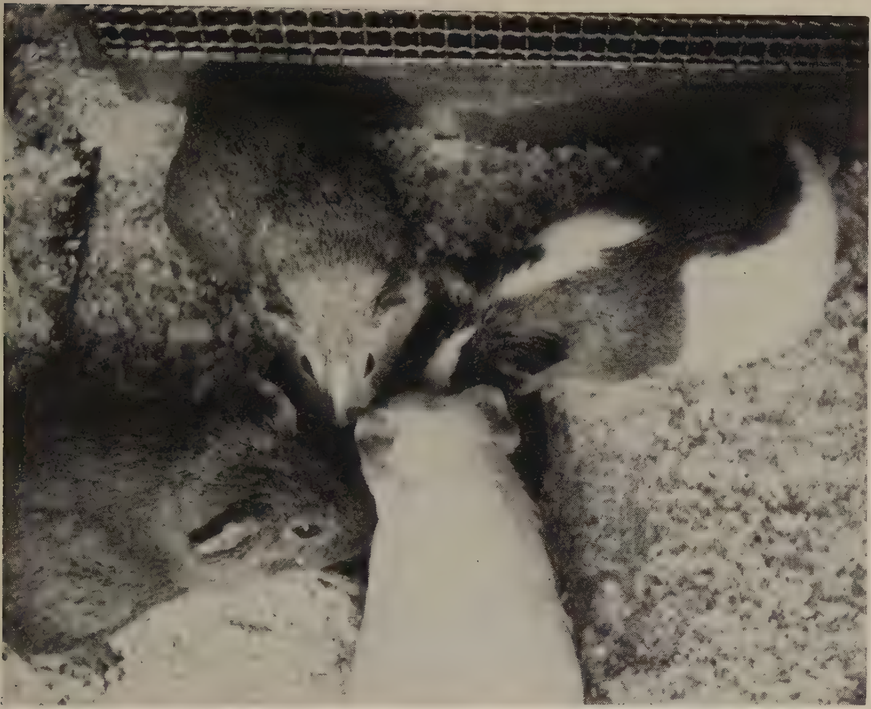

FIGURE 6. Adult germfree guinea pigs. The male (left foreground) was 1 year of age at this time. The other three animals, all females, were 337, 296, and 296 days of age, respectively.

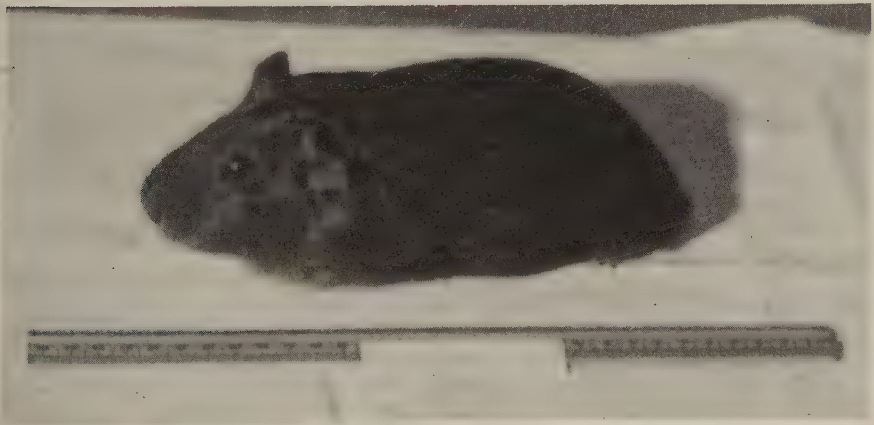

FIGURE 7. Adult guinea pig immediately prior to sacrifice and examination at the age of 408 days. This animal had been maintained germfree for 406 days, at which time it had become accidentally contaminated with *Micrococcus*. A 15-inch ruler is included to show the size of the animal.

The oral cavities were clean and the teeth well developed. Post-mortem examination showed the perioral lymphnodes to be pale and insignificant. The stomach and small and large intestines, with exception of the ceca, were normal. The mesenteric lymph nodes and especially the Peyer's patches were small when compared to conventional standards. The appearance and quantity of gastric and intestinal contents were normal. The most striking departures from conventional characteristics were in the greatly distended ceca. Despite this distension, however, passage was well maintained throughout the intestinal canal, as indicated by observations made *in vivo* and by the normal pellet formation in the large intestines. The pancreas and liver appeared normal. The gall bladder showed good tonus, the bile was clear and contained a small, soft, greenish precipitate. Normal conditions appeared throughout the nervous, respiratory, circulatory, and urinary systems. The spleens were small and showed normal distribution of white and red pulp. The pituitaries and adrenals showed the conventional characteristics. The thymi were small and fatty. The male reproductive organs were normal; viable spermatocytes were observed in abundant quantities in sperm obtained from the epidydimus and various points of the ductus deferens. The ovaries appeared anemic; signs of recent ovulation were not observed. On microscopic examination, numerous ova in various stages of maturation were observed, together with many follicles that showed an early atresia. Corpus luteum tissue was not present. The other reproductive organs of the females appeared normal.

The fact that the animals had not reproduced was a matter of both interest and concern since they were beyond the age at which reproduction occurs in conventional guinea pigs. In this connection, the possibility of vitamin E deficiency was considered even though the animals had shown no signs of muscular dystrophy, which is a symptom of vitamin E deficiency in the conventional guinea pig, during their lifetimes. The post-mortem examinations, however, especially examination of the male reproductive organs, tended to exclude the possibility of vitamin E deficiency. Examination of the ovaries, which revealed a retarded rate of development, perhaps explained the failure of the animals to reproduce. Although numerous ova in various stages of maturation were observed therein, corpus luteum tissue could not be positively identified. No explanation was provided for the failure of ovulation to be completed in the animals but, on the basis of information available from studies of the conventional guinea pig, it was believed improbable that this was related to a deficiency of any known vitamin.

The Problem of Cecal Distension

Due to the comparatively mature status of the guinea pig at birth, the rearing of this animal germfree is not complicated by what may be the most difficult problem attending the rearing of other laboratory mammals under similar conditions. This is the problem of hand-rearing the animals through a suckling period of considerable duration. Rearing of the guinea pig germfree, however, has appeared to be no less difficult than the rearing of other germfree laboratory animals. It has its own peculiar problems, the persistence of which has been responsible for the slow progress that has been made. One of the more persistent of these problems is that of cecal distension and the accompanying difficulties in elimination that this condition seems to produce.

The cecum of the guinea pig is characteristically large as compared to that of certain other laboratory animals. In the conventional guinea pig, its wall is tough and thick, but in the germfree guinea pig it is more elastic and very thin. In the conventional cecum, ingested foods undergo considerable alteration due, in part, to vigorous bacterial activities prior to entering the large intestine. In the germfree cecum, no bacteria are present, and the alteration of ingested materials therein results only from metabolic and digestive activities of the animals. Although the cause of cecal distension in the germfree guinea pig has not been completely established, certain observations have suggested the following sequence of events. Peristalsis, in the thick-walled conventional cecum, forces its content into the large intestine. Similar activity in the thin-walled germfree cecum forces its content to distend the cecal wall. Eventually the quantity of ingested material retained in the germfree cecum increases to such an extent that the organ distends on all sides to the boundaries provided by other tissues and organs. At this time, when there is no available space for further distension, peristalsis becomes more effective in forcing ingested materials into the colon.

Abnormal cecal distension, in varying degrees, is a rather consistent characteristic of germfree mammalian life. It occurs in germfree rats and mice, but presents less of a problem in these species than in the germfree guinea pig. The cause of the condition, presumably the same in all species, appears to be a lack of resistance to stretching on the part of the cecal wall. The germfree guinea pig, if maintained on soft diets of low fiber content, such as our L-445 regimen, lives a seemingly healthy life. Cecal distension is ever present in the animal, however, and is the sole anatomical abnormality of any magnitude we have observed. Cecal distension may remain a normal characteristic of the germfree guinea pig and may be proved, eventually, to be nondetrimental to the health and reproduction of the animals. At the present time, however, we are inclined to consider cecal distension an abnormality and believe that it warrants consideration in any future study on the rearing of the guinea pig germfree.

Hematological Characteristics of Germfree Guinea Pigs

Some representative data obtained by hematological examination of germfree guinea pigs reared on dietary regimen L-445 are presented in TABLE 8A. These data show hemoglobin values and total red cell counts that were not significantly different from the 12 to 18 gm. per cent and 4 to 8 million cu. mm., respectively, that characterized the results obtained from examination of our conventional guinea pigs. The total white cell counts, however, were appreciably lower in the germfree animals than the 5 to 12 thousand cu. mm. range observed in conventional guinea pigs of similar ages. The differential distribution of leukocytes was also significantly different in germfree and conventional animals. The percentage of neutrophils in the germfree guinea pigs was very

TABLE 8

HEMATOLOGICAL CHARACTERISTICS OF GERMFREE GUINEA PIGS MAINTAINED ON DIETARY REGIMEN L-445

Age (days)	Total count			Differential (per cent)					
	Hb. gm./100 ml.	RBC $\times 10^6$	WBC $\times 10^3$	Neutrophils	Eosinophils	Basophils	Lymphocytes	Mononuclear cells	Kurloff* cells
A									
22	16.1	5.19	5.75	51	0	0	43	7	0/100
32	12.4	5.42	1.75	16	1	1	82	0	0/200
36	15.7	6.62	1.80	45	0	0	54	1	0/100
45	16.1	7.52	1.25	40	1	0	55	4	0/200
50	17.1	6.68	1.55	71	0	0	28	1	0/100
50	16.6	6.53	1.15	82	0	0	18	0	0/50
56	13.8	4.96	1.45	80	0	2	17	1	0/100
56	11.7	4.44	2.50	63	0	0	32	5	0/100
63	16.6	3.54	.80	68	0	0	29	2	0/50
70	13.2	6.46	1.00	33	0	0	62	5	0/100
Mean	14.9	5.73	1.90	55			42		0/110
±	0.62	0.38	0.45	6.8			6.7		
B									
30	10.4	7.03	1.00	10	0	0	87	3	3/100
30	10.8	4.59	3.50	22	0	1	71	6	2/100
30	10.3	4.11	2.45	32	0	1	64	3	4/100
36	13.3	5.77	6.70	2	0	0	95	3	21/100
40	11.5	4.57	4.05	4	0	0	94	2	7/100
40	12.3	6.32	3.80	0	0	0	94	6	1/100
40	12.0	4.75	9.50	2	1	1	96	0	6/100
41	11.8	5.08	3.50	5	0	1	88	6	0/100
46	13.2	5.27	1.55	63	0	0	34	3	0/100
71	12.7	5.49	7.80	2	0	1	95	2	8/100
Mean	11.8	5.29	4.48	14			82		5.2/100
±	0.32	0.28	0.87	6.34			6.35		
C									
30	10.9	5.26	3.85	19	1	6	71	3	3/100
30	9.7	4.63	5.30	8	0	8	83	1	2/100
30	10.0	4.21	6.15	11	0	7	73	9	8/100
30	13.5	5.16	6.40	15	0	2	82	1	8/100
36	10.2	4.95	7.30	15	3	5	73	4	3/100
36	9.9	4.44	13.10	3	0	0	90	7	8/100
36	10.6	4.95	4.50	22	0	2	72	4	13/100
40	10.4	4.31	4.45	2	0	0	95	3	6/100
41	6.6	3.41	8.90	12	1	0	85	2	0/100
41	10.8	3.99	6.05	16	1	0	80	3	2/100
Mean	10.2	4.53	6.60	12			80		5.3/100
±	0.53	0.18	0.86	2.05			2.58		

(*A*) Normal germfree animals; (*B*) germfree animals received intracecal inoculation of *E. histolytica-T. cruzi* at age of 12 days. Rare *T. cruzi* were observed in the blood, but amoebic lesions were not present; (*C*) same as in *B* except amoebic lesions were also present in the ceca.

* Number of cells containing Kurloff bodies/number of monocytes and lymphocytes (combined) examined.

high, up to 82 per cent, as compared to 8 to 23 per cent in our conventional animals. The percentage of lymphocytes in the germfree animals was, in most instances, considerably lower than the 76 to 90 per cent range we observed in conventional guinea pigs of similar ages.

On the other hand, germfree guinea pigs that received an intracecal inoculum of material derived from cultures of *Endamoeba histolytica* and *Trypanosoma cruzi* developed certain hematologic characteristics similar to those of our uninfected conventional guinea pigs. This occurred in animals (TABLE 8*B*) that showed a parasitemia with *T. cruzi* but no amoebic lesions, as well as in animals (TABLE 8*C*) wherein amoebic lesions were also observed. This response to protozoan inoculation was characterized by an elevated white cell count and an increased percentage of lymphocytic elements therein. Apparently some activity of the protozoan organisms tended to stimulate a response on the part of the lymphatic system which, in the germfree animal, is characteristically underdeveloped. A similar response sometimes occurred in certain germfree guinea pigs that were not exposed to living microorganisms. Alterations of the blood cellular components were observed to have occurred in germfree guinea pigs that were affected by stresses of nonmicrobial origin. Some germfree guinea pigs with anal prolapse and accompanying hemorrhage and the 2 germfree animals with induced scurvy showed elevated white cell counts and an increased percentage of lymphocytes comparable to that occurring in animals harboring the protozoa. A comparison of the hemoglobin values for normal germfree animals (TABLE 8*A*), with those obtained for germfree animals (TABLE 8*B* and *C*) that received an inoculum of protozoa suggests that these infections may have been accompanied by a lowering of the hemoglobin values. However, additional data may be required before any definite conclusions can be reached in this regard.

The remaining data of significance in TABLE 8 present the results of our search for Kurloff bodies in the germfree guinea pig. These cytoplasmic inclusions, described first by Kurloff (1899), are believed to be present in varying percentages of the monocytes and lymphocytes in all strains of the guinea pig (Smith, 1947). The data show that Kurloff bodies were not observed in our normal germfree guinea pigs. These inclusion bodies were observed, however, in germfree guinea pigs with induced protozoan infections. Furthermore, the percentages of monocytes and lymphocytes containing Kurloff bodies in the inoculated animals were not significantly different from the 2 to 20 per cent range observed in our conventional guinea pigs. Kurloff bodies were not observed in germfree animals affected by stresses of nonmicrobial origin. Even in the 2 germfree animals with induced scurvy, wherein the total white cell count ranged as high as 6800 with a combined distribution of monocytes and lymphocytes of 89 per cent, no Kurloff bodies were observed in 1000 monocytes and lymphocytes examined.

Kurloff bodies were not observed in germfree guinea pigs that had reached or even passed the ages at which these bodies appeared in our conventional guinea pigs of the same strain. Nevertheless, it is not inconceivable that these inclusions may appear later in the life of the germfree animals at a time when the lymphatic system has become more fully developed. In this connection it seems pertinent to point out that 4 Kurloff bodies were observed during examination of 500 lymphocytes from one of our oldest formerly germfree animals. However, although this animal had been maintained germfree for 378 days, it became contaminated with *Micrococcus* approximately 48 hours prior to the time it was examined on day 380 of its life. The question as to what effect the contamination, although of short duration, may have had on the appearance of Kurloff bodies in the blood cannot be answered.

Summary and Conclusions

The rearing of the guinea pig germfree has been studied in experiments extending over a period of 5½ years. Numerous liquid, dry, and semisolid diets were devised and evaluated for their efficacy in sustaining life and promoting growth in the germfree animals. The methodology evolved from the studies permits obtaining germfree guinea pigs of good quality and maintenance of the animals in a state of good health to adult life. The survival time of the animals has been increased from a few days in the early trials to more than a year in our more recent experiments. Some limited information was obtained concerning the vitamin requirements of the germfree guinea pig. Certain anatomical and hematological characteristics of germfree guinea pigs reared by the method of our choice are discussed. We were unable to rear germfree guinea pigs into the second generation in the limited efforts in this direction. However, although reproduction in the germfree guinea pig is a matter of considerable scientific interest, it is of questionable practical importance. The animal is easily obtained germfree by cesarean section and, due to its comparatively advanced state of maturity at birth, requires no hand-feeding or other complicated feeding procedures during the period of its infancy. For these reasons, especially, the first-generation germfree guinea pig may be considered a more practical tool for use in biological investigation than certain other first-generation mammals such as rats and mice.

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The efforts of the following individuals, all of whom contributed either advice or services that aided these investigations, are acknowledged: Bernard Teah, Ervin Zelmer, Anthony Celichowski, Daniel Moran, Merrillyn Tasher, Margaret Beaver, Louise Lowney, Morris Wagner, T. D. Luckey, B. S. Wostmann, and Leonard Knight. All are members or former members of the staff of Lobund Institute. Our appreciation is expressed also to Samuel Poiley, Laboratory Aids Branch, National Institutes of Health, for providing gravid guinea pigs of excellent quality for the cesarean sections.

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MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERIZATION OF GERMFREE LIFE*

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The need for a characterization of germfree life in some detail can be justified in different ways.

First, now that germfree animals have been reared for generations and on a larger scale, we need current information about the general characteristics of the germfree stock. More specifically, we must ask ourselves whether various germfree species, as they are available today, truly represent the standardized biological tool of our long-standing anticipation.

A second reason, stressing the need for descriptive work on the germfree animal, concerns its broad use in the field of experimentation. The germfree host, differing from normal stock not only by its absence of the bacterial flora but also by its lack of systemic responses to that flora, is an animal that is considerably different from the conventional one. The average worker who wishes to use the germfree tool occasionally cannot possibly gauge for himself the details of this broader background, yet it is clear that these details will affect his results. Therefore, it appears that there is need for the elaboration of a limited but comprehensive parameter in this field.

A third justification for descriptive work in the germfree animal concerns the environmental physiologist. A study of the germfree host in comparison to the normal stock, including animals exposed to controlled bacterial contamination, portrays the effects of the normal flora on the animal with great clarity.

Finally, there is genuine appeal in the exploration of this largely unknown form of life.

In view of these considerations, the questions raised in this presentation may be defined as: (1) What are the conspicuous characteristics of the germfree animal as it is reared today in comparison to the normal stock? (2) Are these characteristics indeed caused by the lack of bacterial stimulation, or can it be conjectured that they are the result of other, accidentally introduced, nonbacterial variables, such as the highly artificial environment or diets?

Before taking up the results of our work, a brief review of the history of this problem is needed.

The origin of this discipline is unusual in that the man who conceived it thought that its essence, normal germfree life, was not possible. Pasteur,[1] formulated in 1885 what he himself called a preconceived idea, that life would become probably impossible in animals deprived of their normal flora. In saying this, it was pointed out by some of his contemporaries, Pasteur expressed his belief in the principle of the survival of the fittest. The normal bacterium-haboring animal in nature must necessarily represent some form of

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the fittest, and the flora must be regarded mainly as the animal's synergist. If this were not so, other forms without a flora would have survived in the phylogenetic development.

Pasteur's experimental supporter at the turn of the century was Schottelius.[2-5] Although this investigator contributed much to the methods of rearing germfree chickens, his animals were fed inadequate diets and were therefore unsuited for portraying the effects of the missing bacterial flora.

Opposed to the ideas of Pasteur and Schottelius were those biologists of the day who were impressed by the ability of various normal flora elements to produce substances that are more or less poisonous. In their thinking these substances must necessarily impair the health of the animal. Nencki,[6] in 1886, was the first to attack Pasteur on theoretical grounds. About ten years later Nuttall and Thierfelder[7-9] aimed to prove that Nencki was right with their now classic first experiments on germfree mammals. Again, their few, very young, and inadequately fed guinea pigs offered little in terms of the effects of the flora on the body. However, it was in the course of their work that the following observation was made for the first time: "the cecum was strongly puffed up and filled to overflow with a brown liquid which contained cheese-like coagula." This characteristic of germfree rodents follows, or perhaps we should say persecutes, those who rear germfree animals to this day.

As we know, the most eloquent champion of the idea that the flora can assume the role of the fighting antagonist against the host was Metchnikoff.[10, 11] In proving this point one of his associates, Cohendy,[12] about 1910, succeeded in rearing germfree chickens of remarkable quality, both in terms of growth and general health. Paradoxically, Cohendy performed his work first with Schottelius in Freiburg im Breisgau, Germany, and later at the Pasteur Institute in Paris, France.

Shortly before World War I Küster,[13, 14] a student of Schottelius, was one of the most active workers in the field. However, his contribution was chiefly in the theoretical and technical development of the discipline. It is of interest that he found a nearly identical moisture content in the liver, kidney, and muscle of his germfree and normal-stock young goats. This proved that the weight gain of the germfree animal represented true growth and not retention of water only. From his description we can also conjecture that the intestinal contents of his germfree animals were greatly increased.

The first investigator to undertake intensive research on the germfree animal was Glimstedt.[15] In essence, Glimstedt found a somewhat inhibited development and a lower content of lymphoid tissue in various organs of approximately 30- and 60-day-old germfree guinea pigs. This characteristic was particularly conspicuous in organs that are normally in close association with the flora.

About 1937, Balzam[16] reared healthy germfree chickens without giving, to my knowledge, any closer description of his germfree animals.

I now return to my first question: What morphological and physiological characteristics have we observed in germfree animals to date?

First, some technical details.

In general, our animals were maintained in the steel units of Reyniers *et al.*[17, 18] and occasionally, before sacrifice, in the plastic isolators of Trexler and Reynolds.[19] Chickens were hatched in the germfree environment from germicide-treated eggs. The germfree rodents were third- to eighth-generation descendants of the original cesarean-born ancestors. Both the germfree and normal-stock animals were fed steam-sterilized diets that had been proved adequate for the given species. As a rule, the normal-stock controls were housed in the conventional animal room, special groups of them were kept occasionally in isolator units similar to those already mentioned. The chicken used in this work was the White Wyandotte Bantam and the White Leghorn, the rat an inbred Wistar, and the Swiss albino strain mouse. The ages of these animals was up to one year, with a preponderance of 1- to 3-month-old groups. Thus, in this paper we deal only with animals that were fully adapted to their environment, insofar as the validity of this statement goes. Additional technical details of the experiment are given elsewhere by Gordon *et al.*,[20] and by Reyniers *et al.*[21] Throughout this paper the symbol "M" stands for the arithmetic mean, and "p" for the statistical "p"[22] of the observations.

As a starting point, may I mention that there is nothing unusual in the external appearance of the germfree animal. In terms of growth the germfree (GF) chicken compares favorably to the normal-stock (NS) controls (FIGURE 1).

As mentioned by Wostmann elsewhere in this publication, this is not the case among our rats and mice. The growth of the germfree males is clearly retarded in comparison to that of their controls; among the females this discrepancy is not as conspicuous, but it is still present.

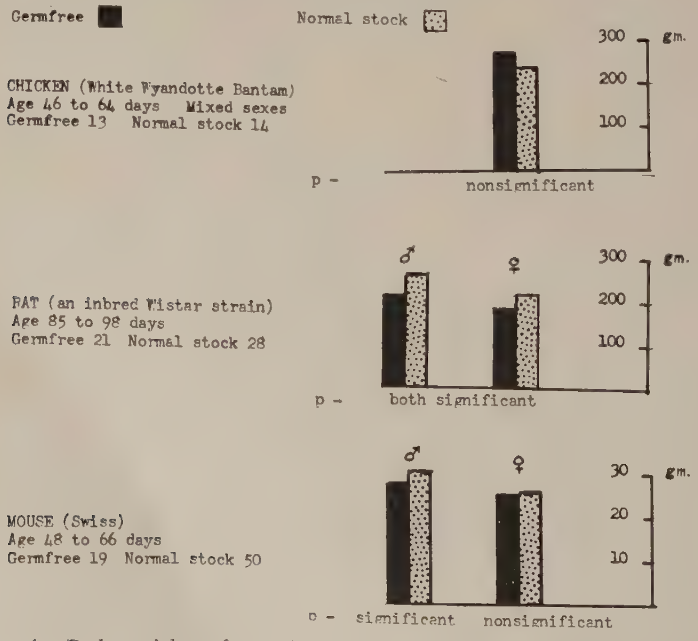

FIGURE 1. Body weights of germfree and normal-stock animals, corrected for cecal contents.

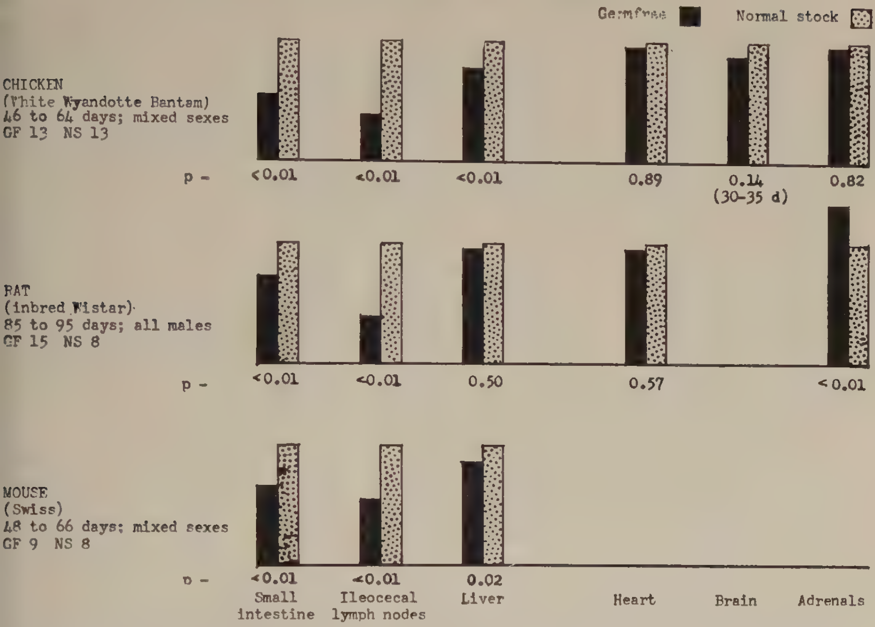

FIGURE 2. Weights of various organs in germfree and normal-stock animals, calculated per unit of body weight (normal stock = 100 per cent).

On autopsy of the germfree animal most details appear normal. However, when the relative weights of various freshly excised organs are compared, certain characteristics become conspicuous (FIGURE 2). Accordingly, in all species hitherto examined, various parts of the digestive tract, notably the small intestine, its associated lymph nodes and, in some instances, the liver, show a lower weight in the germfree group. This, in essence, corroborates the findings of Glimstedt, which I have already mentioned. On the other hand, organs such as the heart or the brain show little or no difference between the germfree and the normal stock. The endocrine glands, for example, the adrenals, clearly align with the second group of organs in the chicken. Incidentally, FIGURE 2 loes not show the similar concentration of ascorbic acid and total cholesterol found in the adrenals of germfree and normal chickens.

In the germfree rat, on the other hand, the adrenals appear heavier. The haracteristics described in FIGURE 2 present themselves during the first weeks of life and persist with certain changes throughout the period of observation. Thus, in terms of weight, the organs of our animals fall into two distinct groups: ne harbors, or is in close association with, an abundant flora in normal life and hows reduced weight in the germfree animals; the other group of organs that s normally remote from bacteria shows practically no difference between the ermfree and the normal stock. Using the expression of Claude Bernard[23] in modified sense, we refer to these two groups as organs of the external or internal environment.

In an attempt to explain the mechanism of this difference we proceeded to the following question. Among organs showing reduced weight in germfree animals, do all elements of such organs participate equally in this reduction, or does this change develop at the expense of one or few elements only? To date we have investigated only a few aspects of this question.

One such aspect concerns the percentage of water in various organs. FIGURE 3 indicates that in the germfree groups the moisture content of some organs of the external environment is slightly but persistently lower than in normal-stock animals. However, it is also clear from this figure that the slight reduction in water content can account only for a small share of the substantially lower weight of these organs.

We next turned our attention to the content of lymphoid tissue of various organs (FIGURE 4). As is well known, the underdevelopment of this tissue in germfree animals has been reported by Glimstedt[15] and more recently, by Gustafsson,[24] Thorbecke *et al.*,[25] Miyakawa *et al.*,[26] and by Phillips *et al.* elsewhere in these pages. Here, in addition to the weight of various organs, we have also determined the amount of lymphocytes per cubic unit of native tissue by means of a method routinely used in our laboratory. The result was again the characteristic dichotomy of the behavior of organs: reduced weight and lymphocyte concentration in organs of the external environment in the germfree group, and nonsignificant changes in organs remote from bacteria, for example, the thymus. The spleen, since it produced variable results in our hands, has not been included in FIGURE 4. Thus, the deficit in lymphoid tissue of the germfree group can account for another aliquot of the lower organ weights under discussion.

The next question to be discussed here concerns the scattered lymphocytes and, more generally, the reticuloendothelial cells of various tissues. TABLE 1 shows the quantitative distribution of such elements in the lower ileum of young germfree and normal-stock chickens, as established with camera lucida and planimeter techniques. These, however, are preliminary results only.

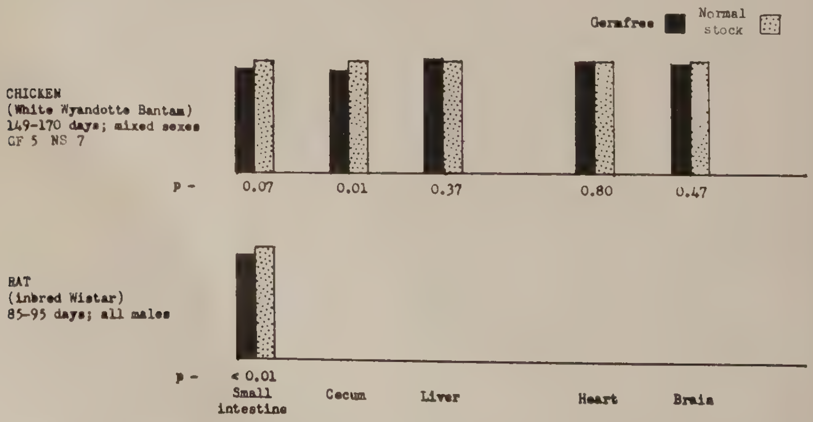

FIGURE 3. Water content (per cent) of various organs in germfree and normal-stock animals (normal stock = 100 per cent).

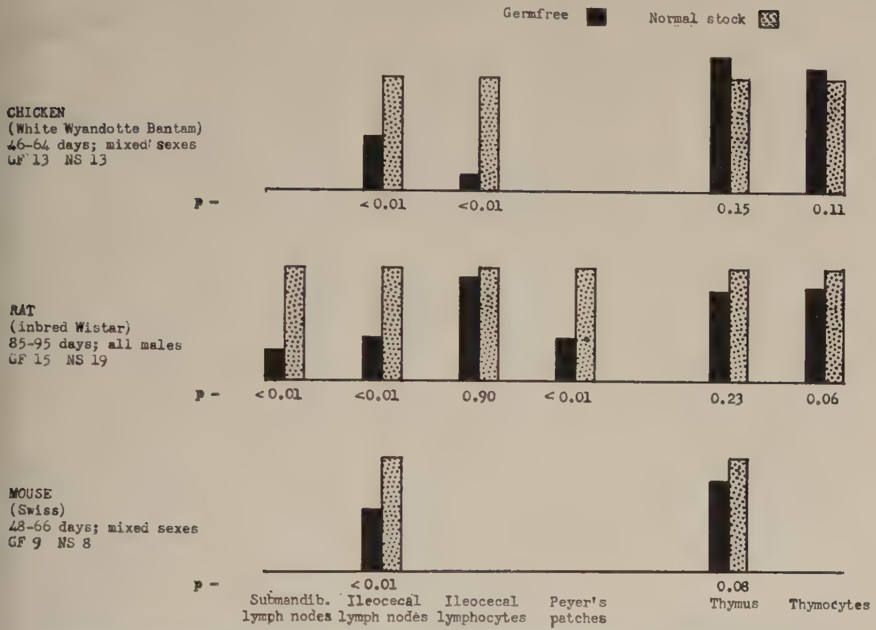

FIGURE 4. Weights and lymphocyte content of various organs in germfree and normal-stock animals. Weights are calculated per unit of body weight, and lymphocytes per cubic unit of tissue (normal stock = 100 per cent).

TABLE 1

SCATTERED RETICULOENDOTHELIAL ELEMENTS IN THE MUCOSA AND SUBMUCOSA OF THE LOWER ILEUM IN GERMFREE AND NORMAL-STOCK WHITE LEGHORN CHICKENS*

(Per Cubic Millimeter of Tissue in 4-μ Sections, Hemalum-Azure-Eosin Stain; Arithmetic Mean of 9 Sections from 3 Animals per Group)

	Epithelium			Lamina propria and submucosa		
	Germfree	Normal stock		Germfree	Normal stock	
Heterophil leukocytes	3	1		15	31	
Eosinophil leukocytes	1	1		10	21	
Basophil leukocytes	7	12		7	11	
Globule leukocytes	116	700	S†	22	16	
Lymphocytes	214	339		520	1010	S
Monocytes	1	4		8	20	
Plasma or blast cells	0	0		26	277	S
Macrophages	1	0		5	23	S?

* Birds of mixed sexes, aged 30 days.
† Significant at 0.05 or less.

Differences between these two groups of animals are demonstrable in terms of the globule leukocyte within the epithelium and in the lymphocytes, plasma cells and, possibly, the macrophages of the lamina propria and submucosa.

For some time we have suspected that the amount of connective tissue present

TABLE 2

PERCENTAGE OF TISSUE DISTRIBUTION IN CROSS SECTIONS OF THE SMALL INTESTINES OF GERMFREE AND NORMAL-STOCK WHITE LEGHORN CHICKENS* AND ALBINO RATS† OF BOTH SEXES

(Iron Hematoxylin and van Gieson Stains)

		Epithelium	Connective tissue	Muscle
		per cent		
Chicken, lower ileum	GF	32.8	20.3	46.8
	NS	30.4	23.8	45.9
Rat, lower ileum	GF	53.6	29.3	17.1
	NS	48.5	34.2	17.3

* Chickens: 30 days old; 13 or 14 sections from 5 animals per group.
† Rats: 242 days old; 5 to 7 sections from 2 animals per group.

in the intestinal canal and other exposed organs is also affected by the presence or absence of the flora. TABLE 2 indicates that, on histological examination, a consistently lower percentage of connective tissue was found in the intestine of the germfree chicken and rat. At the same time, the smooth muscle tissue did not seem to be affected, while the epithelium always appeared with higher values in the germfree.

As we have been taking blood counts routinely during the past years, I have thought it of some value to present these results in TABLE 3, in which the blood of the germfree chicken is shown to be similar to that of the normal-stock bird

TABLE 3

BLOOD CELL COUNTS AND HEMOGLOBIN VALUES IN GERMFREE AND NORMAL-STOCK ANIMALS

		Chickens*		Rats†	
		M	p	M	p
Red blood count $\times 10^6$	GF	3.18		10.2	
	NS	3.17	0.97	8.1	0.01
Hemoglobin, gm. per cent	GF	8.82		—	
	NS	8.99	0.71	—	
White blood count $\times 10^3$	GF	18.14		4.91	
	NS	31.55	0.01	6.71	0.23
Heterophil count (chick) or Neutrophil count (rat) $\times 10^3$	GF	5.87		0.69	
	NS	5.00	0.50	1.36	0.01
Eosinophil count $\times 10^3$	GF	0.15		0.048	
	NS	0.07	0.80	0.099	0.14
Basophil count $\times 10^3$	GF	0.62		0	
	NS	0.74	0.50	0	
Lymphocytes $\times 10^3$	GF	10.62		3.86	
	NS	24.42	0.01	4.61	0.40
Monocytes $\times 10^3$	GF	0.82		0.14	
	NS	1.44	0.03	0.09	0.45

* White Wyandotte Bantam Chickens aged 46 to 64 days, mixed sexes; GF 13, NS 13.
† Rats (inbred Wistar) aged 85 to 95 days, all males; GF 6, NS 9.

TABLE 4

PER CENT DISTRIBUTION OF VARIOUS TISSUES IN THE TOTAL BODY OF GERMFREE AND NORMAL-STOCK WHITE LEGHORN CHICKENS*

(Excluding Feathers, Gastrointestinal Contents, Bile, and Adipose Tissue)

Integument	GF	10.81		Heart	GF	0.69		Cloaca	GF	0.082	
	NS	12.30	S†		NS	0.63			NS	0.089	
Skeleton‡	GF	12.82		Mediastinum‖	GF	0.63		Bursa of Fabricius	GF	0.32	
	NS	11.71			NS	0.70			NS	0.41	S?
Musculature§	GF	54.41		Esophagus	GF	0.37		Liver	GF	3.32	
	NS	53.99			NS	0.34			NS	2.94	
Eyes	GF	1.07		Crop	GF	0.78		Pancreas	GF	0.38	
	NS	0.94			NS	0.69			NS	0.28	
CNS	GF	0.81		Proventric.	GF	0.48		Spleen	GF	0.11	
	NS	0.70			NS	0.52			NS	0.19	S?
Pituitary	GF	0.0009		Gizzard	GF	2.60		Kidneys	GF	1.04	
	NS	0.0009			NS	2.39			NS	1.05	
Thymus	GF	0.36		Small intestine	GF	1.62		Adrenals	GF	0.014	
	NS	0.52			NS	2.20	S		NS	0.015	
Thyroid	GF	0.004		Illeocecal tonsils	GF	0.029		Gonads	GF	0.027	
	NS	0.009	S?		NS	0.049	S		NS	0.069	
Trachea	GF	0.24		Ceca	GF	0.22		Blood¶	GF	5.94	
	NS	0.20			NS	0.33	S		NS	6.01	
Lungs	GF	0.71		Large intestine	GF	0.10					
	NS	0.65			NS	0.08					

* Three GF and 3 NS birds, of mixed sexes, aged 30 to 35 days.

† Significant at 0.05 or less.

‡ Includes only calcified portions and bone marrow as isolated after autoclaving.

§ Includes cartilaginous portions of the skeleton and most of the peripheral nerves.

‖ Includes the large thoracoabdominal vessels and mesenterium.

¶ As obtained by decapitation, including blood and lymph blotted from organs.

except for a low lymphocyte and, possibly, a monocyte absolute count. To date, the rat has offered somewhat different and less clear-cut results.

It next became of interest to know what the distribution of tissues is when we give account of the animal as a whole. The few data we have along these lines for the chicken are presented in TABLE 4. Thus, it appears that we can add the integument to the group of organs that show reduced weight among the germfree. Otherwise most of the other organs display similar weights in our two groups. This includes also the thyroid, whose higher value among the germfree is not significant. In terms of the excess in percentage weight of the germfree animal to compensate for its deficit side of the ledger, we have no clear-cut answer. It seems, however, that we always find a relatively heavier skeleton among the germfree. To date, these are the most persistent characteristics of the germfree animal that we could trace in most of the examined species.

I now propose to consider the question of the causal relationship between our findings and the lack of flora in these animals. This, as you recall, was originally our second question: Are these changes truly the result of absent bacterial stimulation, or is it possible that they are caused by some other unwanted nonbacterial variable that may well have crept into this complex experiment? To this very valid and sometimes tantalizing question we can offer the following evidence and speculation.

(1) Most of the characteristics of the germfree animal develop in a qualitatively similar form, irrespective of species, sex and, limiting ourselves roughly to the first half of life, age.

(2) The described characteristics appear as a plausible result of absent bacterial stimulation.

(3) To the best of our knowledge, the only essential difference between the germfree and the normal control animal is the absence or presence of the contaminating flora. Normal-stock chickens, housed in the steel units of Reyniers (where a special effort was made to maintain the normal flora) and living otherwise exactly under the same conditions as the germfree birds, failed to develop any of the changes that are characteristic of the germfree animal (FIGURE 5).

(4) Germfree stock exposed to reduced environmental stimulation by the lack of the flora can be expected, to display in general reduced variability in its characteristics, at least in organs of the external environment. This, as shown in FIGURE 6, is the case in our best germfree animal, the chicken.

(5) The normal-stock chicken, on ingesting such antibacterial agents as procaine penicillin at low levels from birth (50 mg./kg. diet), will develop a num-

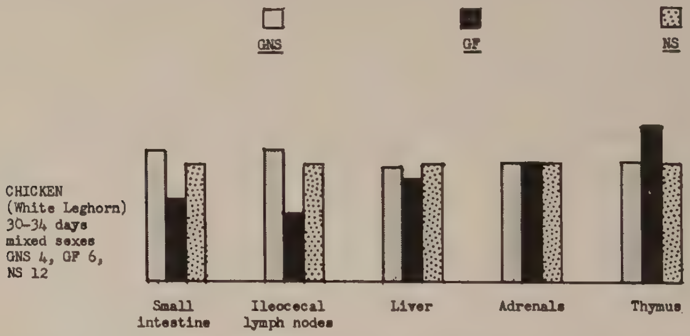

FIGURE 5. Weights of various organs in normal-stock animals housed in germfree-type units and exposed to normal bacterial contamination (GNS) compared to conventionally housed normal-stock animals and germfree animals. Calculated per unit of body weight (normal stock = 100 per cent).

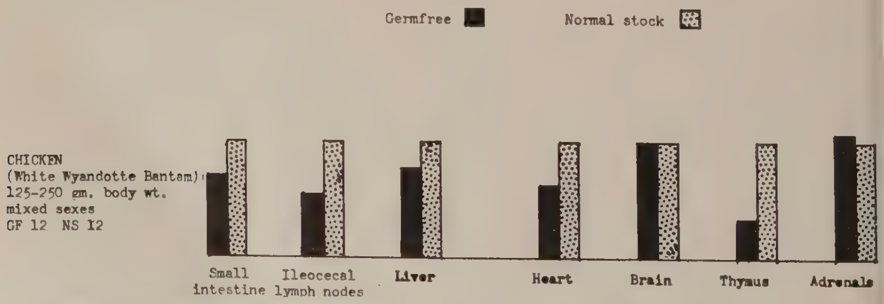

FIGURE 6. Coefficients of variability of various organs in germfree and normal-stock animals, calculated from organ weights per unit of body weight (normal stock = 100 per cent).

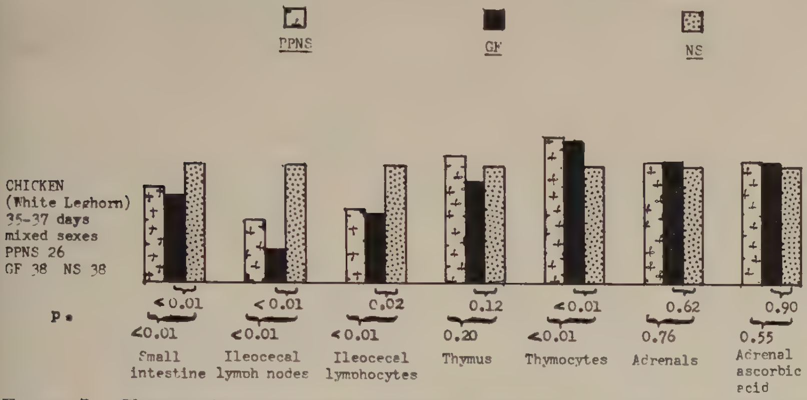

FIGURE 7. Characteristics of procaine penicillin G-treated (50 mg./kg. diet) normal-stock PPNS), untreated germfree animals, and normal-stock animals. Weights are calculated per nit of body weight, lymphocyte content is calculated per cubic unit of tissue, and ascorbic cid is calculated per unit weight of wet tissue (normal stock = 100 per cent).

er of morphological and functional characteristics that are qualitatively simi- ar to those seen in the germfree. As shown in FIGURE 7, the antibiotic-treated ormal-stock animal aligns with the germfree and not with the untreated nor- nal stock.[20] Other indications of functional similarity between these anti- iotic-treated and germfree chickens are offered elsewhere in this monograph y Reyniers in his report on tumor transfer and by Wostmann and Gordon.[27]

(6) Finally, on invasion by the normal flora, the previously germfree animal, emaining on the same diet, will assume most characteristics of the normal tock within a matter of 2 or 3 weeks.

I prefer to discuss separately the enlarged cecum, present mainly in germfree odents and never in chickens, as etiological agents other than bacteria seem o enter into the picture. Almost all workers in this field have observed this henomenon.

In our own experiments the change usually entailed the fourfold to sixfold ncrease of the cecal contents and the twofold to threefold increase in the veight of the cecal sac. This observation was made primarily in adult germ- ree rats and mice normally born in the germfree system and mother-fed. Vhile the presence of this unusual mass in the abdominal cavity does not ffect patently the health of these germfree animals, some of their shortcom- ngs, such as retarded growth and adrenal enlargement, have been brought into ssociation with it.

As to the cause of the cecal enlargement, which appears to be based mainly n reduced muscle tonus, the worker in the germfree field tends to regard the bsent flora as the prime etiological agent. Evidence that proves this latter oint is shown in TABLE 5. Accordingly, on invasion of the normal flora into he GI tract of the now exgermfree rat, the cecal contents begin to reduce in veight within a few hours, while the reduction of the cecal sac lags.

However, it is also known that other nonbacterial agents may be involved the cecal enlargement. Among these are the high starch (notably potato

TABLE 5

WEIGHT OF THE CECAL SAC AND CECAL CONTENTS OF ALBINO RATS*
(Calculated Per Unit Body Weight)

	Germfree	Exgermfree exposed to normal bacterial flora contamination for			Normal stock
		5 to 7 hrs.	1 week	2 weeks	
	Per cent				
Cecal sac	257	260	233	172	100
Cecal contents	665	504	66	79	100

* Male rats (Lobund strain), aged 85 to 98 days: 12 germfree, 40 exgermfree, and 19 normal stock.

starch) content of the diet (Jelinek *et al.*)[28]. FIGURE 8 shows an illustration of bacterial and nonbacterial causes affecting in common the size of the cecum. The combined history of (1) cesarean birth, (2) hand-feeding artificial formulas, and (3) freedom from germs occasionally causes the development of huge ceca in adult rats, up to 30 per cent of the animals body weight with no special handicap in the intestinal passage. The presence of *Lactobacillus* as a monocontaminant in the intestine of cesarean-born hand-fed animals caused a considerable reduction in the weight of the cecum. Even smaller ceca were observed at adult age in normal born and mother-fed germfree animals. Finally, the presence of *Lactobacillus* in the normally born and mother-fed animals seemed to bring the cecum closest to normal dimensions. This indicates that

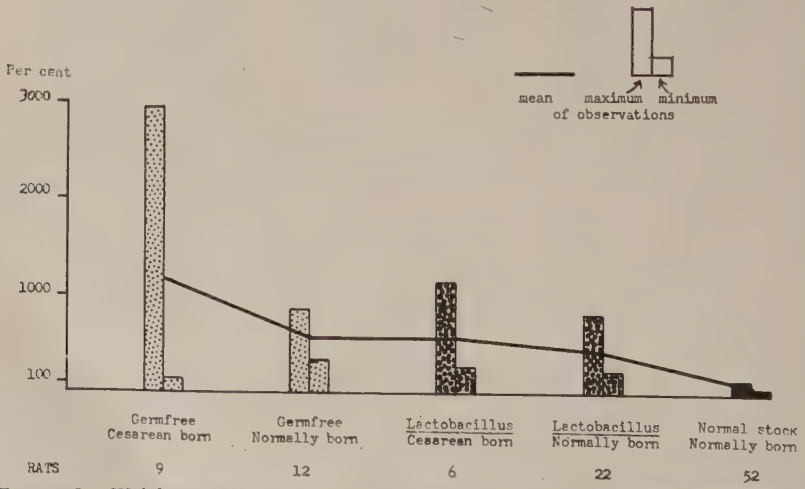

FIGURE 8. Weights of the cecal sac and its contents in germfree, *Lactobacillus*-monocontaminated, and normal-stock albino rats of mixed sex, aged from 62 to 179 days. Normal stock (arithmetic mean) = 100 per cent. Cesarean-born rats were hand fed on artificial formulas and weaned onto semisynthetic diets; normally born rats are mother-fed and weaned onto semisynthetic diets.

at least two factors are involved in the etiology of the enlarged cecum: (1) the flora and (2) an abnormal rearing and feeding history of the animals.

Summary

The presently available germfree chickens, rats, and mice compare satisfactorily to their counterparts in the normal-stock colony in terms of development and general health.

Germfree chickens and rats, in comparison to normal-stock controls, were found to display reduced weight, water, reticuloendothelial tissue and connective tissue content in such organs as are normally in contact with the flora. The same findings, although less extensively tested, were made also in germfree mice. It is assumed that these characteristics of the germfree animal are caused primarily by the lack of the stimulation that would be caused by flora.

The enlarged cecum of the germfree rodents appears to have a more complex etiological background. The slight retardation of growth and adrenal enlargement that was observed in these animals has tentatively been brought into association with this unusual mass in the abdominal cavity.

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THE LYMPHATIC SYSTEM OF GERMFREE GUINEA PIGS

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A germfree animal may be defined as one that has been born and raised under sterile conditions so that it has had no contact with bacteria and thus has never experienced any bacterial invasion of its tissues. It is a matter of great nterest to determine the effects of such a germfree life on lymphatic tissues.

Methods

The guinea pigs that we use are of the Gifu uniform strain obtained by brother-sister breeding. We have obtained and reared germfree guinea pigs using the M-51 and M-56 germfree systems that I described previously[1] and elsewhere in this monograph. However, the rearing capacity of both of these systems is limited to 10 animals at one time, so that it is impossible to maintain a sufficient supply of animals more than 100 days old for the purpose of examining their lymphatic tissues. Consequently, we have reared the germfree animals until they are 40 to 50 days of age and then supplied several animals to several experimental groups, the results of which are supplemented by data obtained from an experiment on germfree animals more than 50 days old. Some of our germfree guinea pigs had a series of specific symptoms, such as alopecia, prolapsus ani, and hernia. These we consider due to the deficiency of essential nutritional elements, probably caused by a disturbance of ingestion and resorption in the bowel; therefore we have excluded these animals as unsuitable for the experiments. Kobayashi fluid diet NG-27 (TABLE 1) is given as soon as the young are born, on the fifth day after birth Kobayashi solid diet NG-36 (TABLE 2) is mixed with the diet NG-27, and at the end of the first month the NG-36 diet solely is used. NG-36 solid diet has been found satisactory for the long-term rearing experiment, but NG-27 diet, a liquid formula or young guinea pigs, still needs improvement before it can be used satisactorily. Therefore a comparison has been made with respect to the growth within 20 days of birth between germfree guinea pigs reared on the Kobayashi diet and natural guinea pigs reared with the same diet. The result indicated hat the germfree animals grow more slowly than those reared in a natural nvironment. After 30 days of age the average increasing rate of body weight per day of the germfree animal becomes parallel with that of the natural animals reared on the Kobayashi diet. Because there was a handicap of growth during the first 20 days of life, the body weight of the germfree animals is lways less than that of normal animals of the same age. Keeping this fact n mind, we have made efforts to reduce the body weight of the control animals o the level of the germfree ones. Both germfree and control animals were ept in the same environmental conditions, that is, all experimental animals were reared at a temperature of 25° ± 3° C. and in the humidity range of 50 ± per cent. We also used a considerable number of natural animals as a pre- minary experiment, as well as the paired germfree and control animals.

TABLE 1

COMPOSITION OF NG-27 DIET (KOBAYASHI) PER 300 GM. OF RATION

Whole dry milk	100 gm.
Aq. dest	200 ml.
Soy beam oil	1.5 ml.
Panvitan (vitamin complex)	3 pellets
Folic acid	7.5 mg.
Vitamin K	7.5 mg.
Vitamin B	30.0 μg.
Biotin	300.0 μg.
Choline	0.15 gm.
Yeast extract	1.5 gm.
Liver extract	75.0 mg.
Inositol	300.0 mg.
PABA	15.0 mg.
Thiamine	27.0 mg.
Riboflavin	6.0 mg.
Pyridoxine	6.0 mg.
Vitamin C	20 to 25 mg./2 days

COMPOSITION OF NG-36 DIET (KOBAYASHI) PER 100 GM. OF RATION

Roasted soy bean flour	70.0 gm.
Sucrose	9.0 gm.
Gum arabic	12.0 gm.
Salts mix	7.0 gm.
Panvitan (vitamin complex)	1 pellet
Folic acid	2.5 mg.
Vitamin K	2.5 mg.
Vitamin B	15.0 μg.
Biotin	100.0 μg.
Choline	0.3 gm.
Yeast extract	0.5 gm.
Liver extract	25.0 mg.
Inositol	100.0 mg.
Pantothenic acid-Ca	10.0 mg.
PABA	5.0 mg.
Thiamine	9.0 mg.
Riboflavin	2.0 mg.
Pyridoxine	2.0 mg.
Vitamin C	25 mg./2 days

Results

The experimental results were as follows.

First, we made a preliminary investigation on many conventional animals, ranging in development from fetus to adult, in order to discover the essential states of the lymphatic apparatus in the nasal cavity. For this purpose the examinations were made by serial frontal section of 50 animals and sagittal section of 4 animals. The results reveal the fact that the anatomical structure of the nasal cavity in the guinea pigs is complicated. A prenatally formed lymphatic apparatus on the central line of the floor of the nasal cavity extends from the posterior edge of the foramina palatina to the position of the fourth molar. Most of the lymphatic tissue contains postnatally formed secondary nodules with clear centers designated by Hellman[2] as *Reactionszentrum*. For instance, in the 40-day-old conventional guinea pig, well-developed lymphatic tissue was found in the nasal cavity, wherein not only solid secondary nodules but also clear-centered secondary nodules, called Flemming-type nodules,[3] were formed. The ciliated epithelium in the mucous membrane became stratified (FIGURES 1 to 4). In germfree guinea pigs of the same age, similar lymphatic tissue and occasional solid secondary nodules were found at the nasal floor, but no clear-centered nodules (FIGURES 1, 2, 5, and 6). In an attempt to investigate the lymphatic tissue in the sinus maxillaris, this cavity was examined in 30 natural animals ranging in development from fetus to adult, serial section specimens being made. The results of the examination revealed that the sinus maxillaris of the guinea pig is a complicated cavity that is not completely encircled by bone, but is open on both sides. As FIGURE 7 shows, distinct secondary nodules were formed in the tunica propria of the mucous membrane of the sinus maxillaris in 40-day-old control animals, and clear centers were recognizable in the nodules. The ciliated epithelium of the mucous membrane of sinus maxillaris has also become stratified. However, in germfree animals of the same age neither secondary nodules nor even lymphocytic infiltrations were found in the tunica propria of the sinus maxillaris; therefore a monotonous picture is seen. The ciliated epithelium covering the sinus maxillaris was arranged regularly in a single layer (FIGURE 8).

Second, we examined the number of Peyer's patches in the cecum of the following 103 natural animals: 50 cases from birth to 20 days of age, 10 cases about 40 days of age, and 43 cases 150 to 200 days of age. It was revealed that 6 to 25 patches were observed in the young animals shortly after birth, 8 to 15 in 40-day-old animals, and 8 to 16 in the 150- to 200-day-old animals. The maximum number of patches in each group was 12 in the young animals, 11 or 12 in the 40-day-old ones, and 13 in the 150- to 200-day-old animals; thus, it ranged from 11 to 13. This fact indicates that the number of Peyer's patches is settled before birth.[4,5] However, the examination of 17 cases of more than 40-day-old germfree guinea pigs has shown the number of Peyer's patches to be from 6 to 8. However, it is not clear whether the smaller number has any significant meaning. Histologically, only a few secondary nodules with clear centers were found in the Peyer's patches in the cecum of the natural young shortly after birth, but great numbers of the nodules were found in the 40- to 200-day-old natural animals. For instance, in the Peyer's patches of a 40-day-old control guinea pig one can see that a distinct and clearly bordered secondary nodule with clear center has appeared (FIGURE 9). On the other hand, in the Peyer's patches of a 40-day-old germfree guinea pig, as may be seen in FIGURE 10, secondary nodules with clear centers were not found, and the Peyer's patch itself is very poorly developed.

Third, in regard to lymph nodes, a preliminary investigation has been made on these nodes in popliteal, abdominoinguinal, mesenteric, and cecal areas of 50 of the young animals shortly after birth, 9 of 40- to 50-day-old ones, and 39 of 150- to 200-day-old animals. As result of the examination of the lymph nodes of 211 germfree animals we can say that the lymph nodes in germfree animals develop more poorly than do those in natural guinea pigs. It is con-

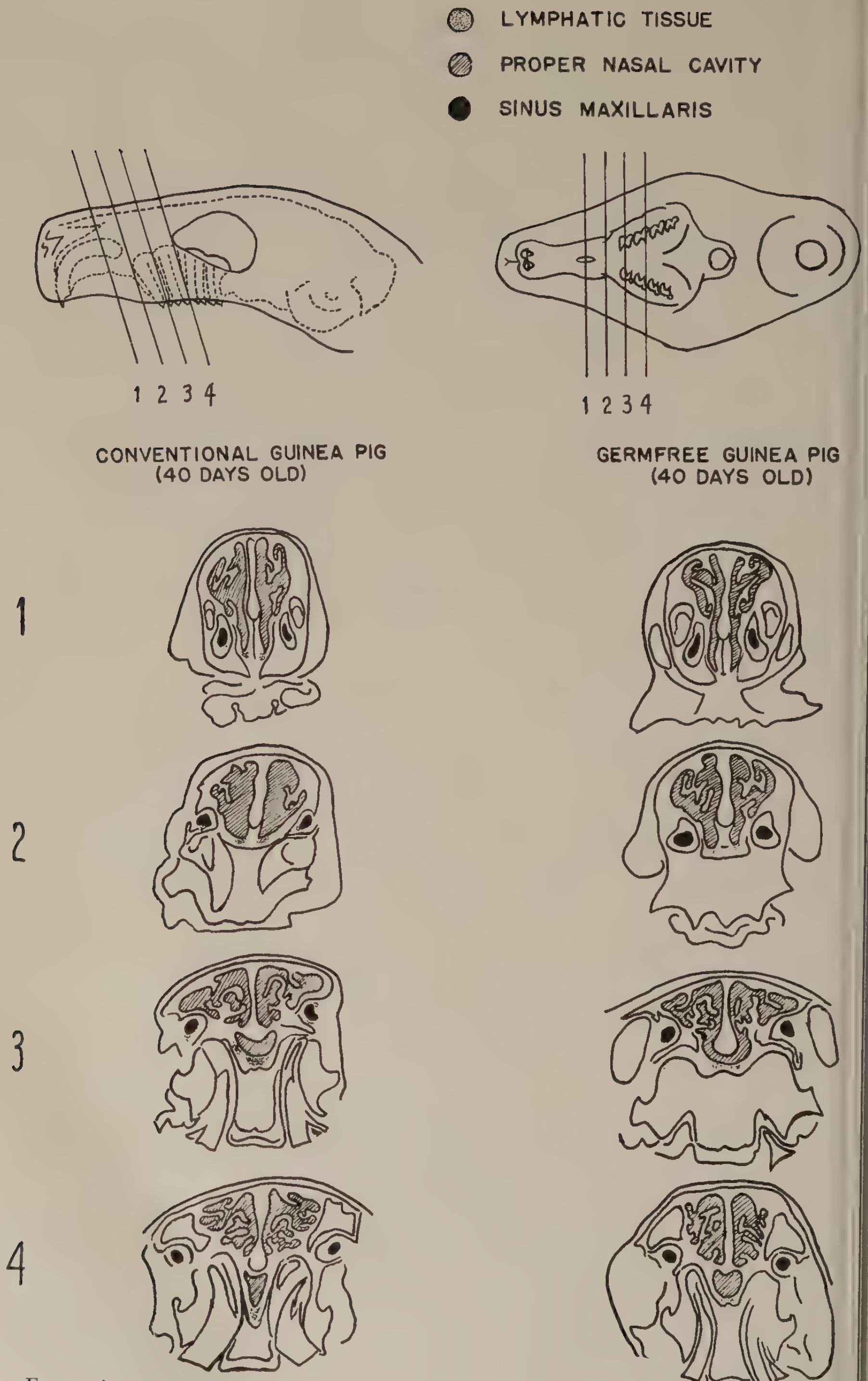
LYMPHATIC TISSUE
PROPER NASAL CAVITY
SINUS MAXILLARIS
1 2 3 4
1 2 3 4
CONVENTIONAL GUINEA PIG (40 DAYS OLD)
GERMFREE GUINEA PIG (40 DAYS OLD)
1
2
3
4

FIGURE 1. Frontal sections of the skulls of conventional and germfree guinea pigs, showing the lymphatic tissue of the nasal cavity and the sinus maxillaris.

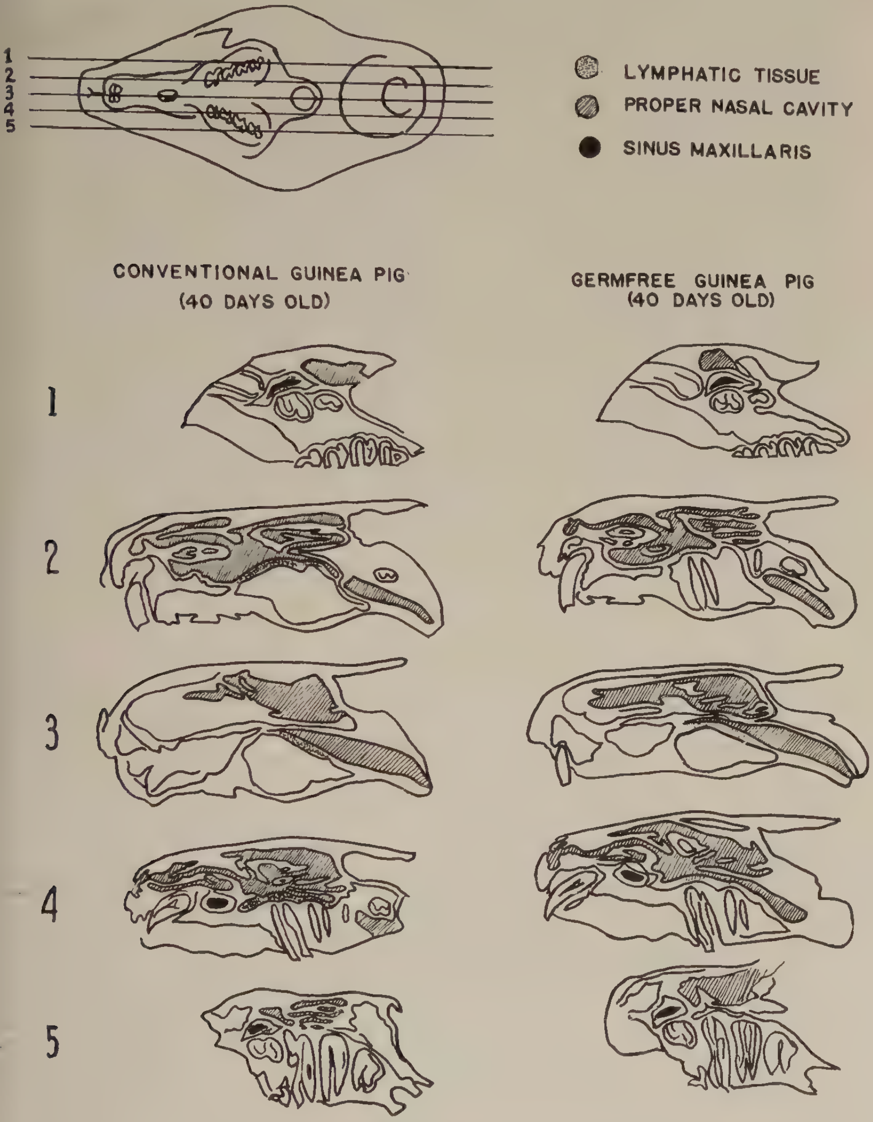

FIGURE 2. Sagittal sections of the skulls of conventional and germfree guinea pigs, showing the lymphatic tissue of the nasal cavity and the sinus maxillaris.

sidered that the mesenteric and cecal lymph nodes have different functions in relation to the intestinal tracts. Even though they are germfree, these animals could not be exempted from the influence of stimulation originating from food in the bowel. Histologically, we could recognize solid secondary nodules, but few secondary nodules with clear centers in the natural guinea pig shortly

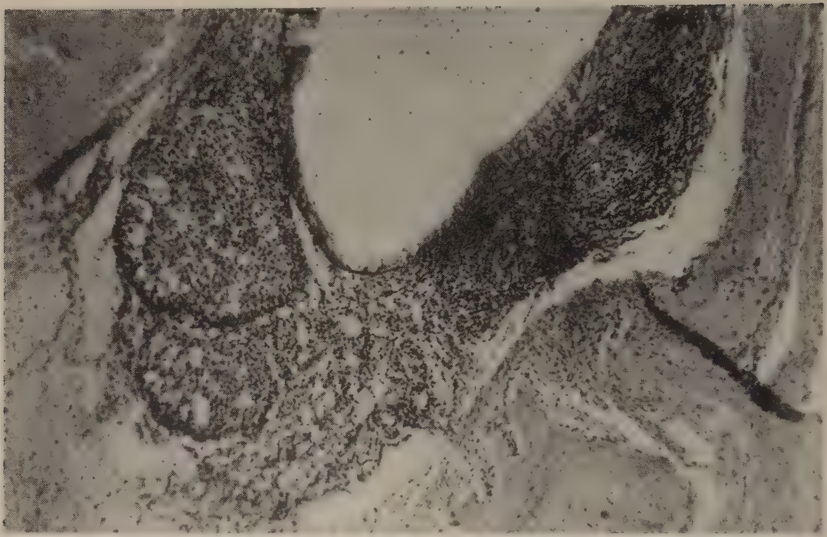

Figure 3. Frontal section of the skull of a conventional guinea pig. Note the well-developed lymphatic tissue in the tunica propria of the nasal cavity. Distinct clear-centered secondary nodules are visible. Hematoxylin-eosin stain. ×280.

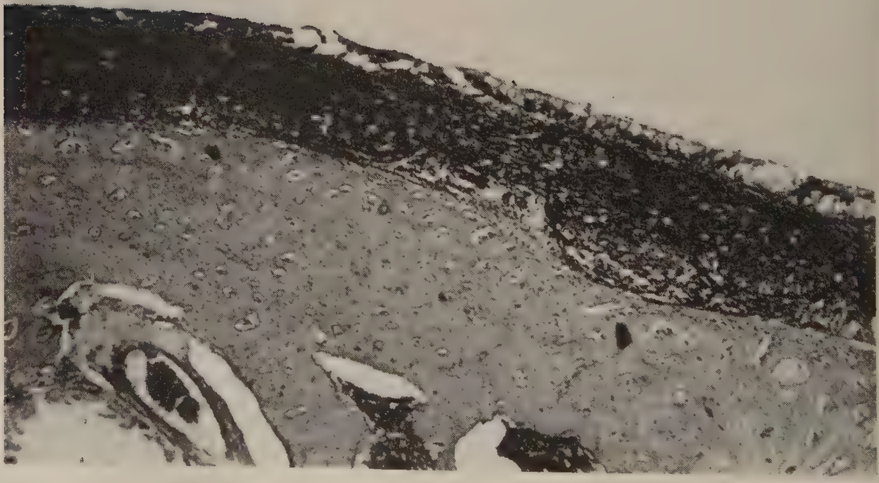

Figure 4. Sagittal section of the skull of a conventional guinea pig. Note the well-developed lymphatic tissue in the tunica propria of the nasal cavity. Clear-centered secondary nodules, as well as solid ones, are visible. Hematoxylin-eosin stain. ×280.

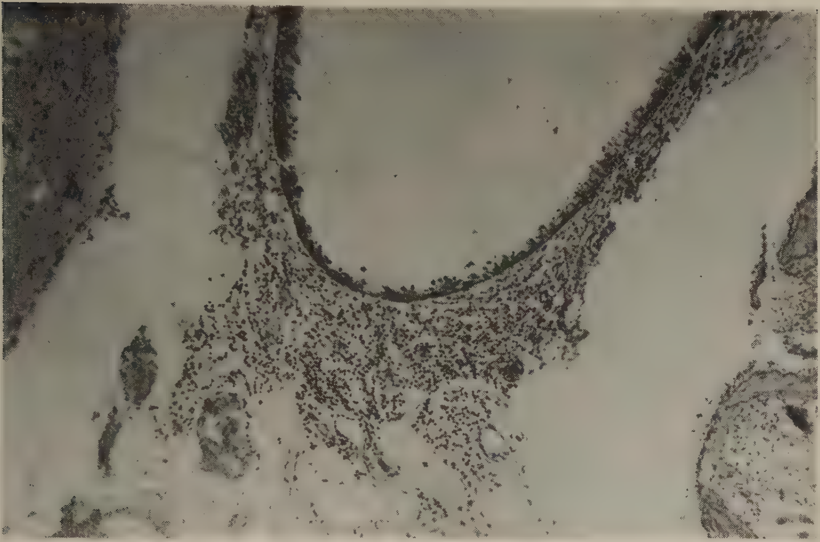

FIGURE 5. Frontal section of the skull of a germfree guinea pig. Note the poorly developed lymphatic tissue in the lamina propria of the nasal cavity. No clear-centered nodules are visible. Hematoxylin-eosin stain. ×280.

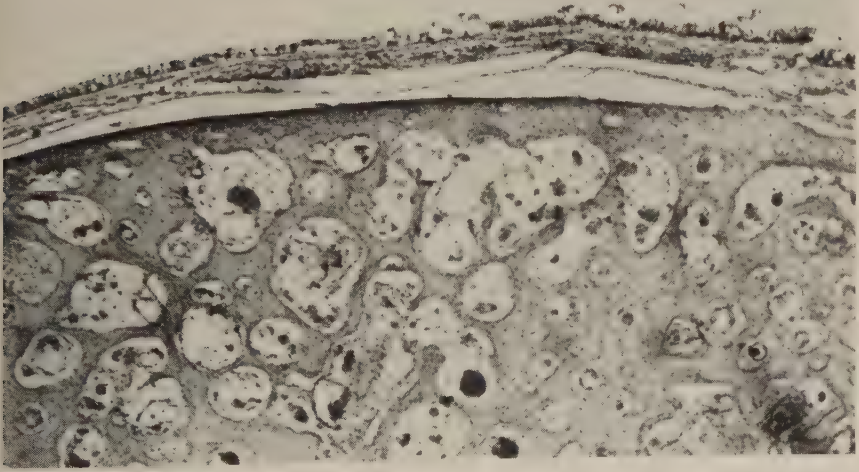

FIGURE 6. Sagittal section of the skull of a germfree guinea pig. Note the poorly developed lymphatic tissue in the tunica propria of the nasal cavity. No clear-centered nodules are visible. Hematoxylin-eosin stain. ×280.

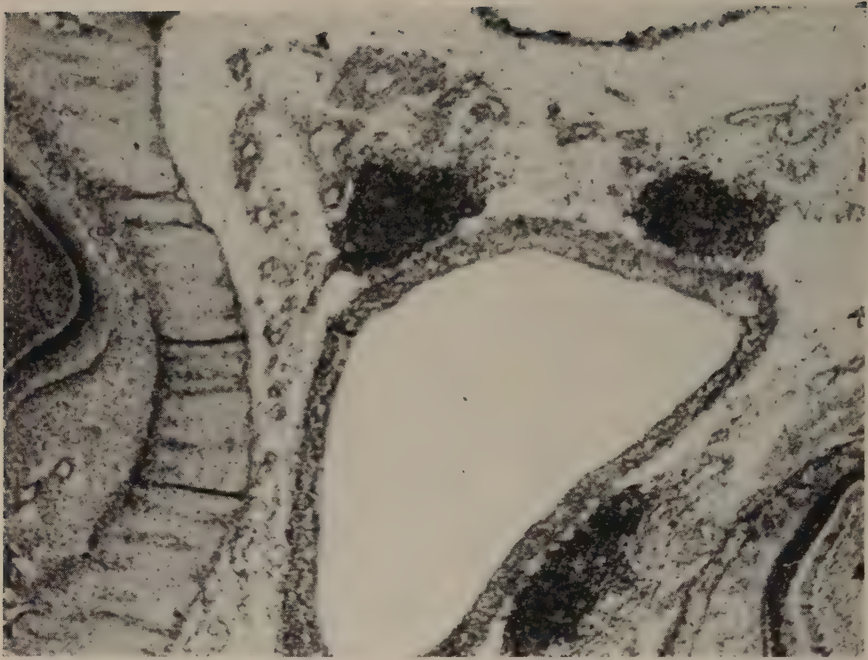

FIGURE 7. Frontal section of the skull of a conventional guinea pig, showing distinct secondary nodules in the tunica propria of the mucous membrane of the sinus maxillaris. Note the clear-centered nodules. Hematoxylin-esoin stain. ×280.

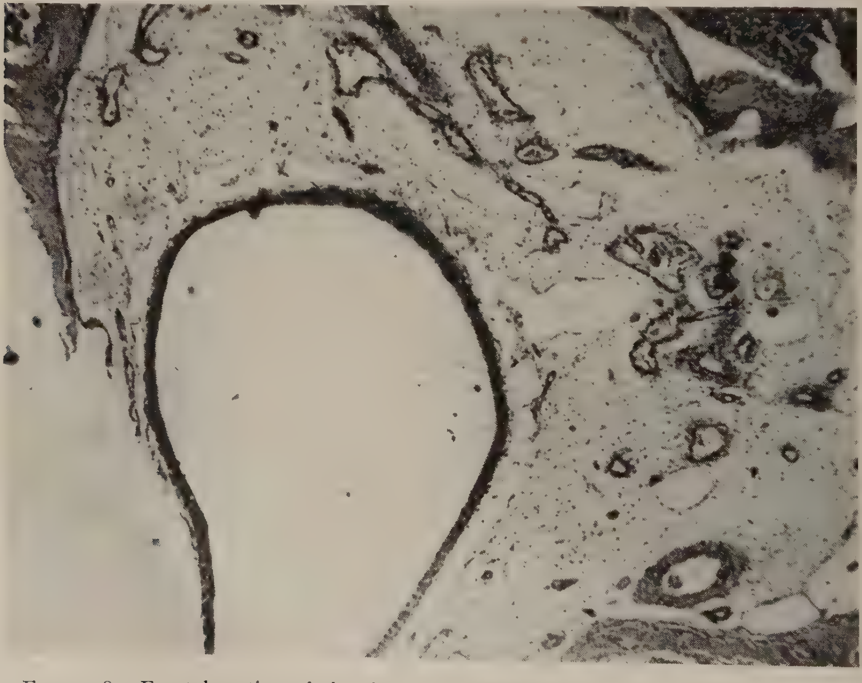

FIGURE 8. Frontal section of the skull of a germfree guinea pig. Neither secondary nodules nor even lymphatic infiltration are visible in the tunica propria of the sinus maxillaris. Hematoxylin-eosin stain. ×280.

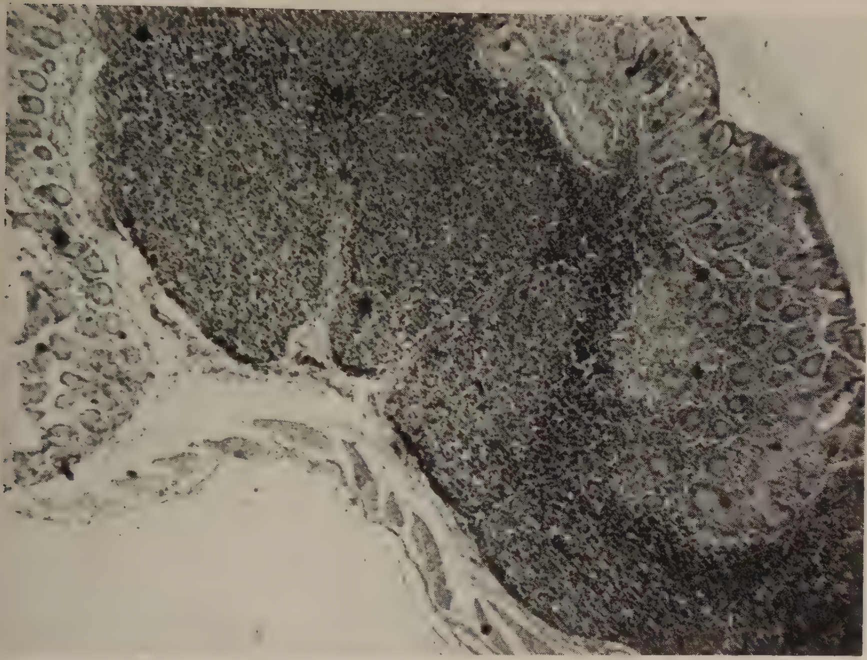

FIGURE 9. The cecum of a 40-day-old conventional guinea pig. Note the well-developed Peyer's patch and the clear-centered nodules. Hematoxylin-eosin stain. ×280.

after birth. However, in the 40- to 200-day-old animals we could find numerous clear-centered secondary nodules, particularly in the mesenteric and cecal lymph nodes. In the 40- to 200-day-old animals, some of the cases have shown that pyronin-stained large cells and typical Marschalko-type plasma cells appear in the medullary cords. For instance, in the popliteal lymph node of a 40-day-old natural control guinea pig, not only solid secondary nodules but also clear-centered secondary nodules on the cortex and numerous pyronin-stained large cells and typical plasma cells on the medullary cord were observed (FIGURE 11). On the other hand, in the popliteal lymph node of a 40-day-old germfree guinea pig, no clear-centered secondary nodules were found, but plasma cells appeared on the medullary cord (FIGURE 12).

Let us now consider the behavior of the lymph nodes in the cases contaminated by single species of bacteria. We have made an investigation of 19 guinea pigs that were monocontaminated because of a failure in the germfree unit during the rearing process. Nine of these animals were contaminated by a single species of spore; the other 10 were contaminated by a single species of *Coccus*. Five succumbed to a bloody watery diarrhea and, in 3 of the 5 deaths, similar involvement was observed in the cecum. Macroscopically, emphysema, necrosis, and bleeding were observed; microscopically, the breakdown of Peyer's

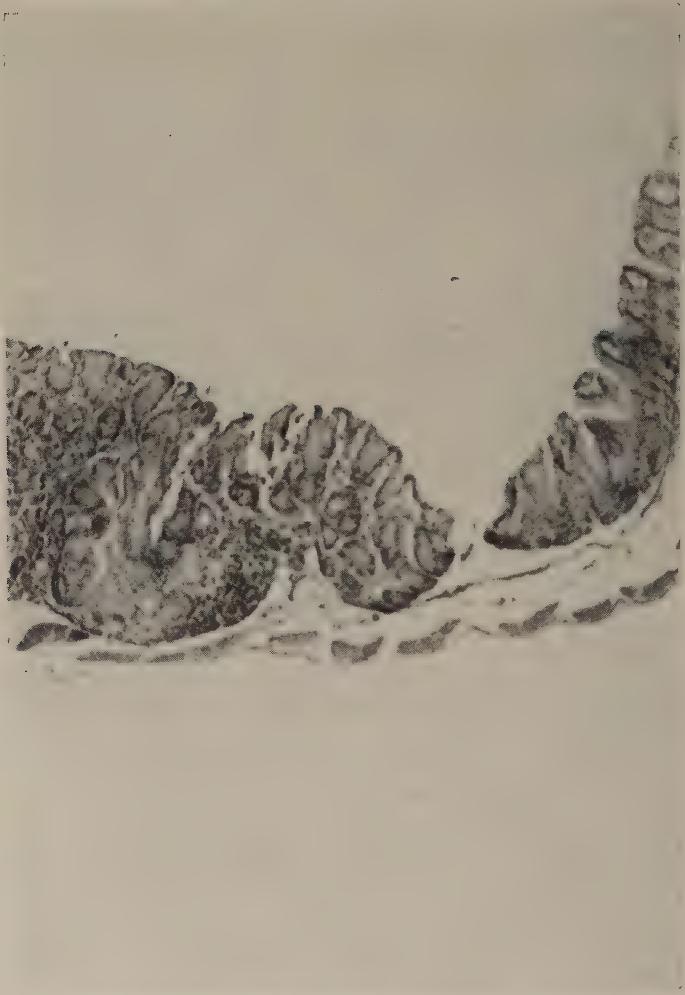

FIGURE 10. The cecum of a 40-day-old germfree guinea pig. Note the poorly developed Peyer's patches and the absence of clear-centered nodules. Hematoxylin-eosin stain. ×280.

patches into numerous small pieces by air vesicles was seen in the wall of the cecum (FIGURE 13). Secondary nodules with clear centers were also found in the cecal lymph nodes (FIGURE 14). Numerous large-sized cells with large nucleoli were observed by making stamp specimens of the node. However, the origin of the large cells cannot be determined. Marschalko-type plasma cells were often detectable on the stamp specimen of the lymph nodes of the germfree animals, as shown in FIGURE 15. However, no abnormalities occurred in the conventional animals that were challenged by the same single species of

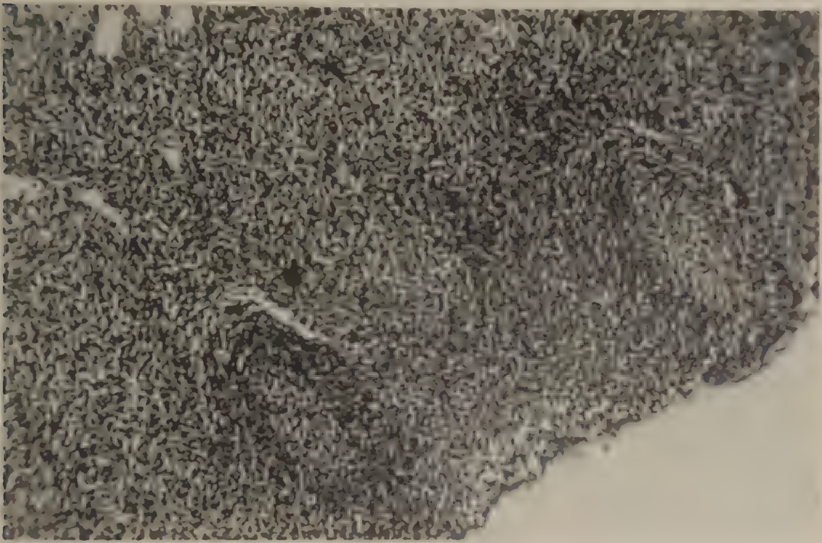

FIGURE 11. Popliteal lymph nodes of a 40 day-old conventional guinea pig. Note the solid and clear-centered nodules. Hematoxylin eosin stain. ×280.

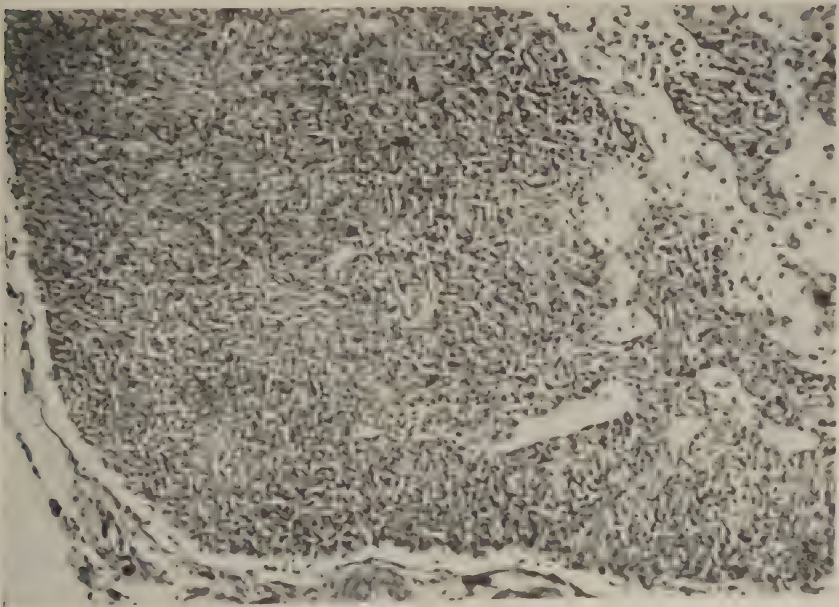

FIGURE 12. Popliteal lymph node of a 40 day-old germfree guinea pig. Hematoxylin-eosin stain. ×280.

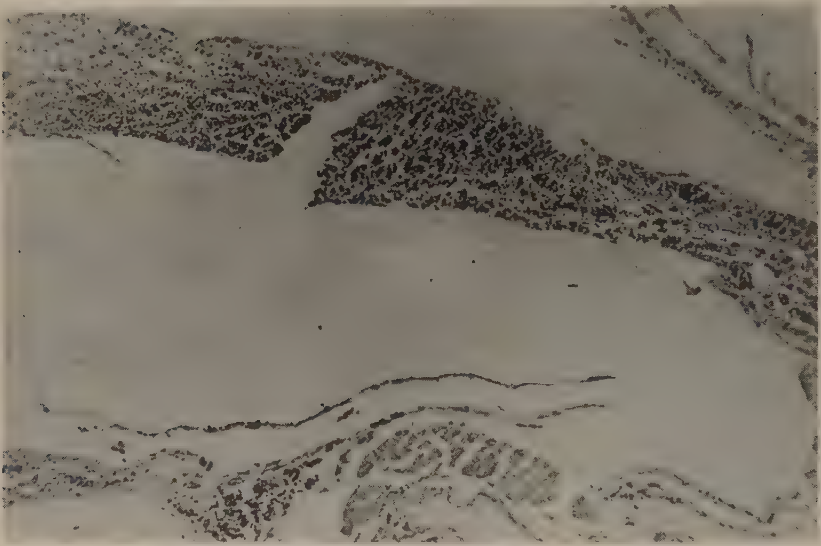

FIGURE 13. Cecum of a germfree guinea pig contaminated by a single species of spore. Note the breakdown of the Peyer's patch into small fragments by air vesicles. Hematoxylin-eosin stain. ×280.

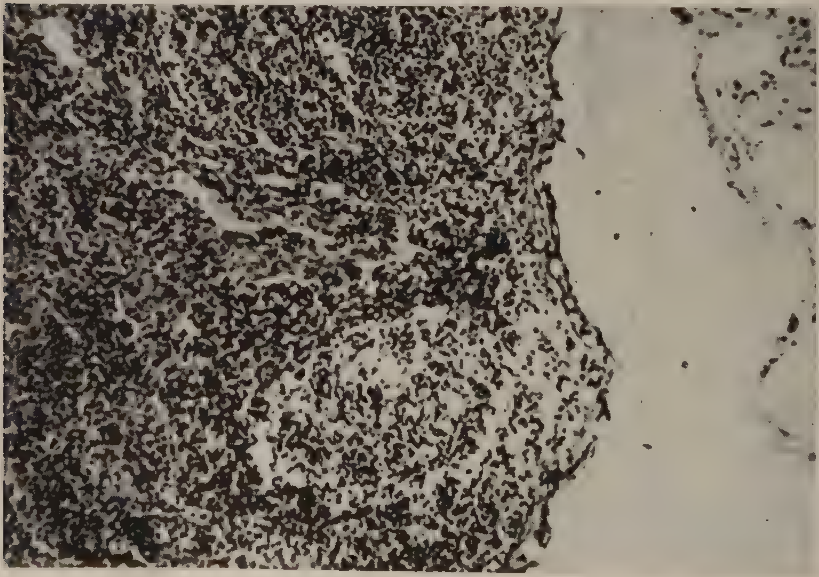

FIGURE 14. Cecal lymph node of a germfree guinea pig contaminated by a single species of spore. Note the appearance of clear-centered nodules. Hematoxylin-eosin stain. ×280.

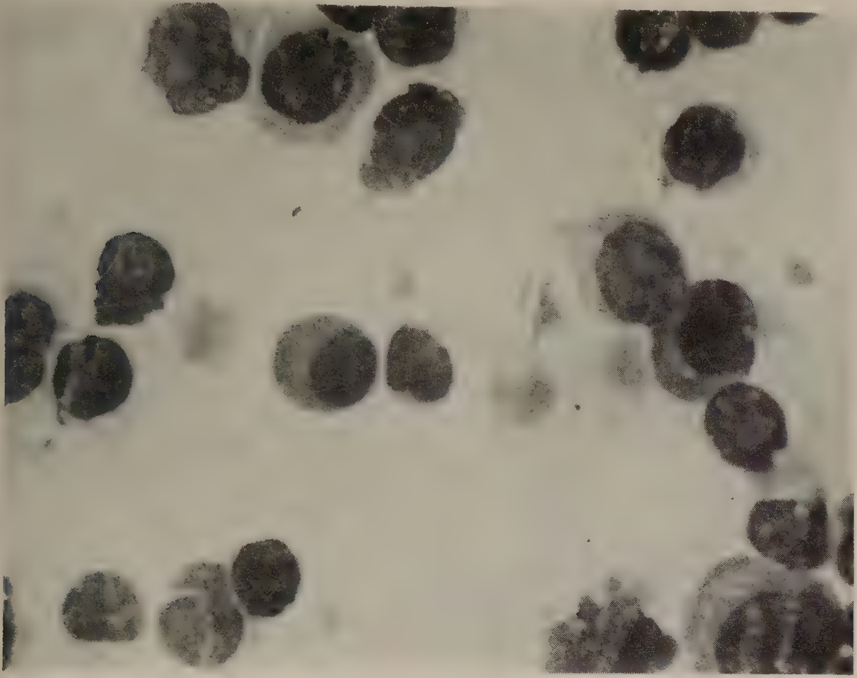

Figure 15. Stamp specimen of the cecal lymph node of a germfree guinea pig contaminated by a single species of spore. Note Marschalko's plasma cells. Hematoxylin-eosin stain. ×900.

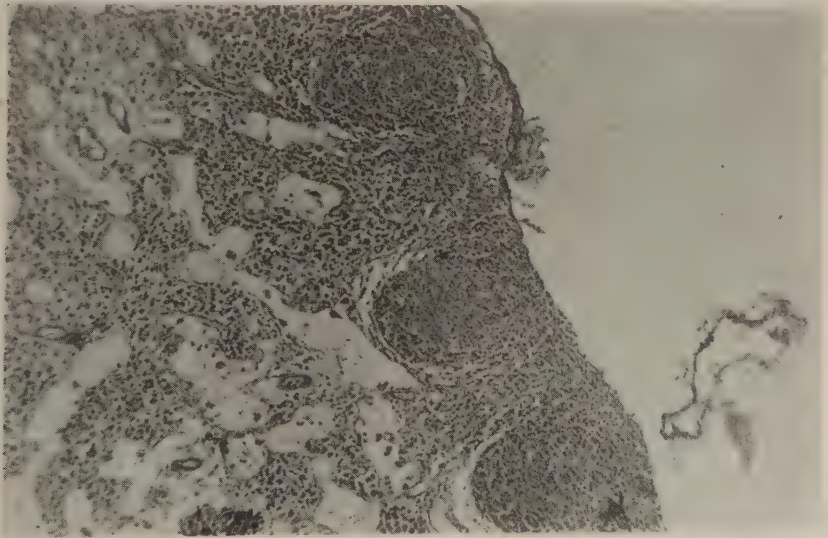

Figure 16. Abdominoinguinal lymph node of a 40-day-old germfree guinea pig contaminated by a mild species of *Coccus*. Note the bare, clear centers without peripheral dark rings. Hematoxylin-eosin stain. ×280.

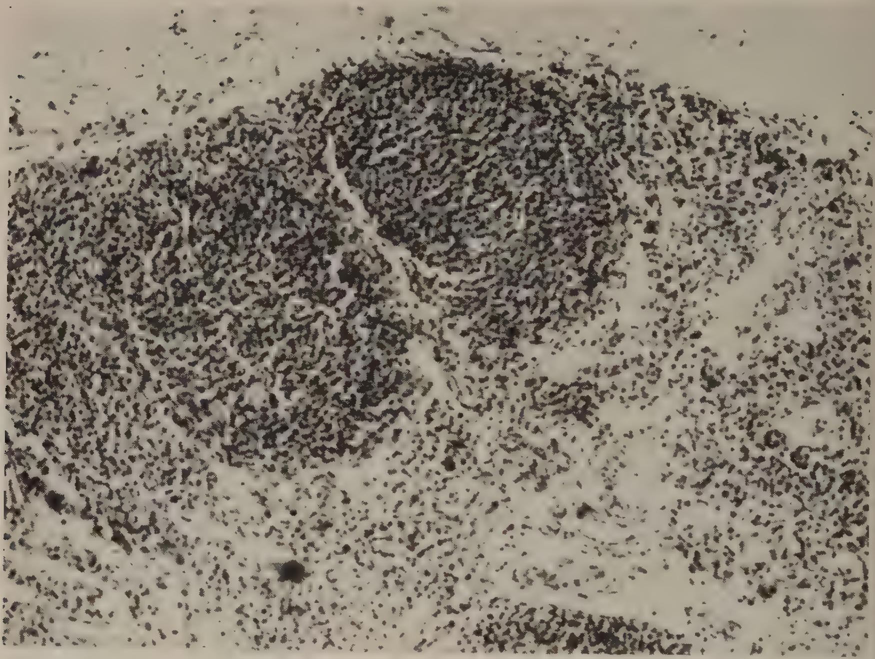

FIGURE 17. Popliteal lymph node of a conventional guinea pig that had been injected in the foot pad with 10 mg. sterilized egg albumin. Distinct clear-centered nodules are visible. Hematoxylin-eosin stain. ×280.

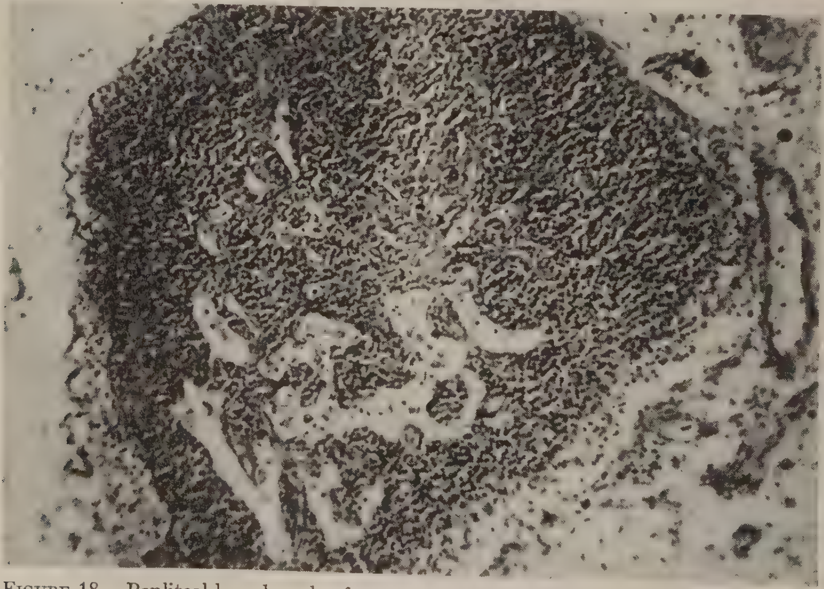

FIGURE 18. Popliteal lymph node of a germfree guinea pig that had been injected in the foot pad with 10 mg. sterilized egg albumin. Hematoxylin-eosin stain. ×400.

spore. It seemed to us that the result described above has indicated the existence of an antagonism between certain microbes in the natural world; in other words, certain bacteria that do not cause pathogenicity under the antagonisms in the natural conventional animal appear to cause pathogenicity in a monocontaminated germfree animal. An investigation of the lymph nodes in the germfree guinea pigs monocontaminated by mildly virulent *Coccus* has revealed that numerous secondary nodules with clear centers and without distinct dark rings appeared. For instance, in the abdominoinguinal lymph node of the 40-day-old germfree guinea pigs contaminated by a mild species of *Coccus*, numerous bare clear centers without peripheral dark rings were apparent. They are like those described as bare germinal centers by Conway[6] (FIGURE 16). Consequently, the results of the experiment indicate that some contamination by mildly toxic bacteria such as cocci contributes essentially to the production of the clear center in the lymphatic tissue. Next, we examined the lymph nodes of germfree animals injected with sterilized egg albumin. Preceding this observation, a preliminary investigation of the changes in lymph nodes of the 66 conventional guinea pigs injected with egg albumin was made; this revealed the fact that the clear-centered secondary nodules on the cortex increased in number and that plasma cells on the medullary cord were prominent on the injected side on the sixth to tenth day after injection as compared with the noninjected side when the precipitation reaction was positive. For instance, 40-day-old conventional guinea pigs that have been injected with 10 mg. of sterilized egg albumin in the foot pads have shown distinct secondary nodules with clear centers and dark rings in the popliteal lymph node in 48 hours (FIGURE 17). After a similar procedure, 40-day-old germfree guinea pigs have shown no nodules with clear centers, but numerous large pyronin-stained cells of diffuse appearance with a large nucleolus that contains ribonucleic acid have been observed. It would be interesting to learn whether these cells gather together to form a nodule (FIGURE 18).

Conclusion

We have thus learned that the development of lymphatic tissue in the germfree animal is less advanced than in the conventional animal. Insofar as we have been able to discover, typical clear-centered nodules have never been found in the lymphatic tissue of germfree animals, but such nodules have been produced most easily by monocontamination by a mildly toxic species of *Coccus*. Since lymphatic tissue is widely considered as the main site of antibody formation,[7-10] the gamma-globulin value of the serum and the state of antibodies such as precipitin and agglutinin in the treated germfree animal are of definite interest.

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SOME HISTOLOGICAL AND FUNCTIONAL ASPECTS OF LYMPHOID TISSUE IN GERMFREE ANIMALS

I. MORPHOLOGICAL STUDIES*

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Animals raised under germfree conditions can be considered to have much less contact with antigenic materials than animals raised in a normal environment. Since it is known that lymphoid tissue is concerned with the defense mechanism against infections, it can be expected that histological differences between germfree and control animals in this tissue will be found.

In this paper I present the results of a comparative histological study of germfree and control animals. Particular attention was given to the occurrence of plasma cells and of secondary nodules in the lymphoid tissue.

Materials and Methods†

The germfree animals used in these experiments were raised in the Lobund Institute, University of Notre Dame, Notre Dame, Ind., according to procedures that are standard there.[2, 3] The methods of testing for bacteriological sterility were developed by Wagner, who describes them elsewhere in this monograph. The restrictions for the term germfree, therefore, that Wagner has discussed apply to the germfree animals used in these experiments. The control animals were housed in ordinary brooders and were raised on the same autoclaved diets as the germfree animals.

The germfree rats used were born naturally from germfree parents within the germfree units and descended through several generations from cesarean-born hand-fed germfree ancestors. They were mature animals of either sex, weighing from 200 to 300 gm. Observations were made on germfree, conventional, and monocontaminated‡ rats that had been transferred from the germfree units to control environments 1 to 4 weeks before sacrifice.

In the case of the chickens, a semisynthetic diet was used that was considered to contain fewer obviously antigenic substances than the crude routine diets. Young Leghorn chickens were studied at the ages of 2, 4, 6, 8, and 12 to 14 weeks.

The organs studied in particular were the spleen, the thymus, the bursa of Fabricius, and the lymphoid tissue of the intestinal tract in the chickens, and the spleen, the mesenteric lymph nodes, and the lymphoid tissue of the intestinal tract in the rats.

The organs to be examined were fixed in a mixture of Zenker's fluid, for-

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† For a more complete description of the methods used see Thorbecke *et al.*[1]

‡ These rats were monocontaminated with a slow-growing, fastidious, unidentified organism that seemed to have very little influence on the animals.

Table 1

Estimation of the Frequency of Occurrence of Plasma Cells (Pl.C.) and Secondary Nodules (S.N.) in Spleen, Thymus, and Ileocecal-Colic Junction of 4-Week-Old Germfree and Control Chickens

Animal No.	Spleen		Thymus		Ileocecum	
	Pl.C.	S.N.	Pl.C.	S.N.	Pl.C.	S.N.
Germfree						
410-6	2	0	0	0	0	0
410-7	3	0	1	0	0	0
410-8	1	1	1	0	0	0
456-1	2	0	1	0	0	0
456-2	2	0	0	0	0	0
456-4		0	0	0	0	0
Control						
509	4	3	2	0	3	2
513	3	2	0	0	4	4
514	3	2	2	0	4	4
515	3	2			4	4
516	3	3	2	0	2	3

Table 2

Estimation of the Frequency of Occurrence of Plasma Cells (Pl.C.) and Secondary Nodules (S.N.) in Spleen, Thymus, and Ileocecal-Colic Junction of 8-Week-Old Germfree and Control Chickens

Animal No.	Spleen		Thymus		Ileocecum	
	Pl.C.	S.N.	Pl.C.	S.N.	Pl.C.	S.N.
Germfree						
410-16	4	2			2	2
410-17	2	0			0	0
456-6	2	0	2	0	0	0
456-7	3	0	1	0		
456-8	2	2	2	0	0	0
Control						
551	3	2	3	0	4	4
552	3	2	3	0	4	4
589	3	2	2	0	3	3
592	4	4	4	0	4	4
596	4	3	3	0	3	3

maldehyde, and 2 per cent trichloroacetic acid (90:5:5) for a period of 5 to 6 hours. The sections were stained with methyl green-pyronin.

An attempt was made to estimate the greatly varying numbers of plasma cells and secondary nodules in the spleen, thymus, and ileocecal-colic junction of the germfree and control chickens of various age groups. An arbitrary scale, ranging from 0 to 4, was established for each organ separately (TABLES 1 to 3).

TABLE 3

GAMMA-GLOBULIN CONTENT OF SERUM AND PLASMA CELLS AND SECONDARY NODULES IN LYMPHOID ORGANS OF GERMFREE, MONOCONTAMINATED, AND CONTROL CHICKENS

Status	Age (weeks)	No. of animals	Average γ-glob. content (per cent)*	Age (weeks)	No. of animals	Plasma cells†			Secondary nodules†		
						Spleen	Thymus	Ileocecum	Spleen	Thymus	Ileocecum
Germfree				2	3	2	0	0	0	0	0
	4	5	9.6 ± 2.1	4	6	2	1	0	0	0	0
	5	5‡	10.7 ± 0.7								
	6	1	12.3	6	3	3	2	2	2	0	1
	8	4	12.5 ± 2.7	8	5	3	2	0	1	0	0
	11	5‡	9.3 ± 1.4								
	14	3‡	8.7 ± 0.9	14	3‡	2		0	0		0
	17	4‡	10.2 ± 0.8								
Monocontaminated	4	10‡§	11.4 ± 1.0								
	12	2‖	14.1; 8.7	12	2‖	2	3	2	0	0	1
Controls				2	5	2	0	2	1	0	2
	4	3	15.8 ± 1.7	4	5	3	2	3	2	0	3
	4	10‡	13.6 ± 1.0								
	5	8‡	15.1 ± 1.0								
	6	3	13.1 ± 1.2	6	3	3	3	3	2	0	3
	8	5	15.9 ± 1.8	8	5	3	3	4	3	0	4
	11	4	18.6 ± 1.9								
	11	5‡	20.7 ± 1.6								
	14	2	40.4; 25.9	14	2	4	4	4	4	4	4
	17	11‡	33.3 ± 2.8								

* Standard deviation of the mean or actual data given.

† The numbers listed represent the arithmetic means of the values (0–4) used in this paper to portray the frequency of the elements found in the spleen, thymus and ileocecal-colic junction.

‡ Animals raised on diet L-291; all the other animals raised on semisynthetic diet L-435.

§ Animals monocontaminated with unidentified bacteria one week before termination of experiment.

‖ Animals monocontaminated with *Bacillus subtilis* 4 weeks before termination of experiment.

Results and Discussion

Before describing the results of these studies it is necessary to define the histological terminology to be used. Throughout this paper, the solid, round-to-oval accumulations of small- and medium-sized lymphocytes, as they are found in the lymphoid tissue of mammals, are referred to as primary nodules, while those lymphoid nodules that have developed a so-called reaction or germinal center are termed secondary nodules.[4] These centers pass through various stages of development. If they are filled with many medium-sized and some large blast cells showing numerous mitoses and some scattered phagocytes, they are called "active centers." In the more inactive centers—often called "clear"— reticulum cells, phagocytes, and a few scattered lymphocytes are visible.

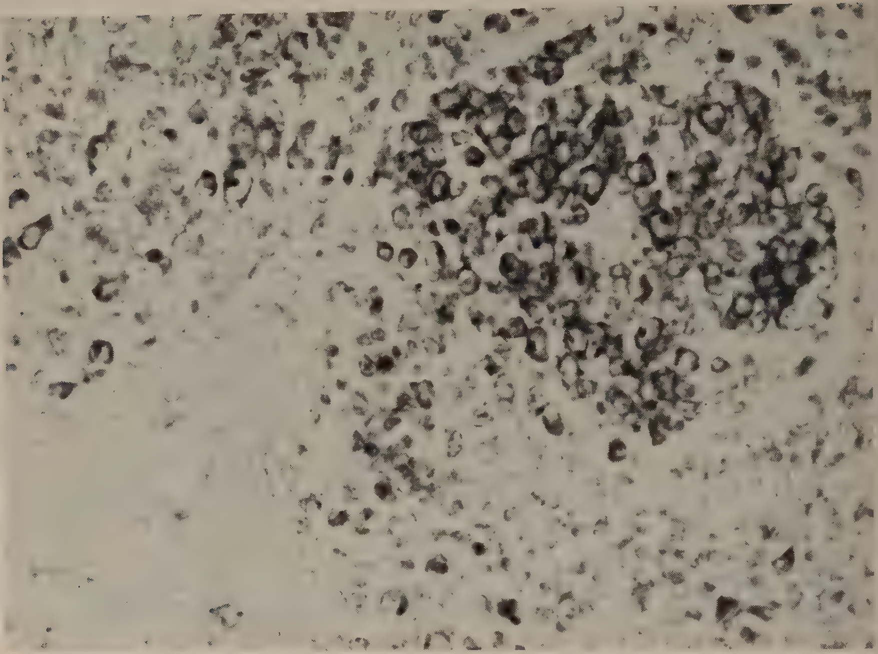

FIGURE 1. Spleen of 2-week-old control chicken: secondary nodule next to the fork of a branching artery. Notice the absence of a zone of small lymphocytes. ×250 (approx.).

So-called "bare" centers, without a dense zone of small lymphocytes around them, are occasionally observed in the lymphoid tissue of mammals.[4, 5] In the chickens, however, in which lymph nodes are absent and the lymphoid tissue is spread much more diffusely throughout different organs, the only real lymphoid nodules observed are structures that show exactly the same morphology as these bare centers of secondary nodules in mammals; that is, they never have a dense zone of small lymphocytes around them. These bare centers in the lymphoid tissue of the chicken, therefore, are termed secondary nodules. They are always adjacent to an arteriole (FIGURE 1).

It has been shown by several investigators that secondary nodules arise when the lymphoid tissue is actively concerned with the defense against foreign substances, notably against invading bacteria.[6, 7] Various observations,[8-12] however, have led to the belief that secondary nodules do not form antibodies, but that immune substances are produced by basophilic blast cells that develop in aggregates closely associated with lymphoid tissue and often located around small arterioles or beneath the epithelial lining of intestines and bronchi. Many of these cells, when matured, show the typical morphology of plasma cells, and it is mainly during this process of cell differentiation that the antibodies are formed.[9] In this paper these cells are referred to as plasma cells and as immature plasma cells.

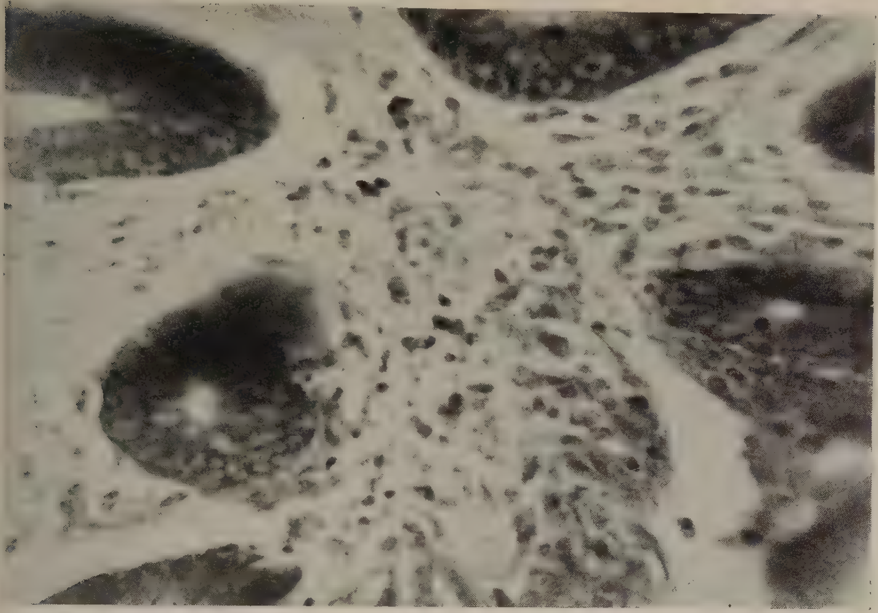

FIGURE 2. Appendix of 8-week-old germfree chicken: arbitrary spot showing very few plasma cells in the stroma beneath the epithelial lining. ×200 (approx.).

Chickens. In chickens the localization of plasma cells is usually the same as in mammals, except in the spleen, where they are located around the sinusoids in the red pulp instead of around the terminal portion of the sheathed arterioles.

An evaluation of the numbers of plasma cells and secondary nodules in the organs of germfree and control chickens (TABLES 1 to 3) shows that there is a quantitative difference in the occurrence of both structures, but that neither of them is completely absent in all germfree chickens. The most conspicuous differences were noted in the lymphoid tissue of the intestinal tract (FIGURES 2 and 3). Up to the age of 6 weeks neither plasma cells nor secondary nodules were visible in any of the sections of the ceca or other parts of the intestines in the germfree chickens used in these experiments; in the control animals quite a few plasma cells and secondary nodules were found by the age of 2 weeks. Gordon, elsewhere in this monograph, states that he has seen a few plasma cells in the stroma of the intestinal wall in 4-week-old germfree chickens; however, his birds were raised on a different diet.

Secondary nodules were absent in the spleens of germfree chickens up to the age of 6 weeks. The numbers of plasma cells in the spleens of germfree chickens were smaller than in the spleens of control chickens in all age groups.

Among the 8-week-old germfree chickens there was one that showed more plasma cells and secondary nodules than any of the others. This illustrates

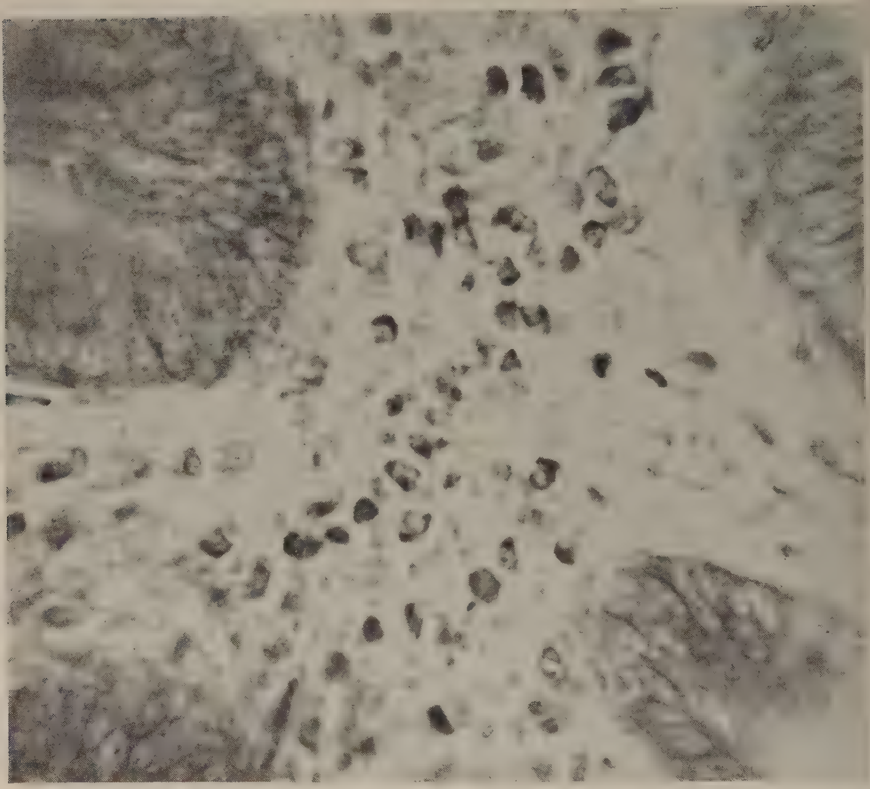

FIGURE 3. Appendix of 8-week-old control chicken: arbitrary spot showing numerous plasma cells in the stroma beneath the epithelial lining. ×275 (approx.).

the variation present in this respect even among germfree chickens in the same cage. FIGURE 4 shows a secondary nodule that occurred in the spleen of such an 8-week-old germfree chicken.

Neither large plasma cell aggregates nor secondary nodules are present in the thymus of mammals. An unexpected finding, therefore, was that in the chicken the thymus showed an increasing number of plasma cell aggregates, first apparent at the age of 4 weeks and, after the age of 12 weeks, of secondary nodules. In this organ there was also a quantitative difference in the number of plasma cells between germfree and control animals.

The histology of the bursa of Fabricius has been described elsewhere.[1] No differences were observed in the size or histological features of this lymphoid organ between germfree and control chickens of the same age. We can probably conclude that the follicles of the bursa of Fabricius differ not only in their morphology from other lymphoid nodules, but also somewhat in their function. These follicles are probably more analogous to the parenchymal tissue of the thymus than to any other lymphoid organ known.

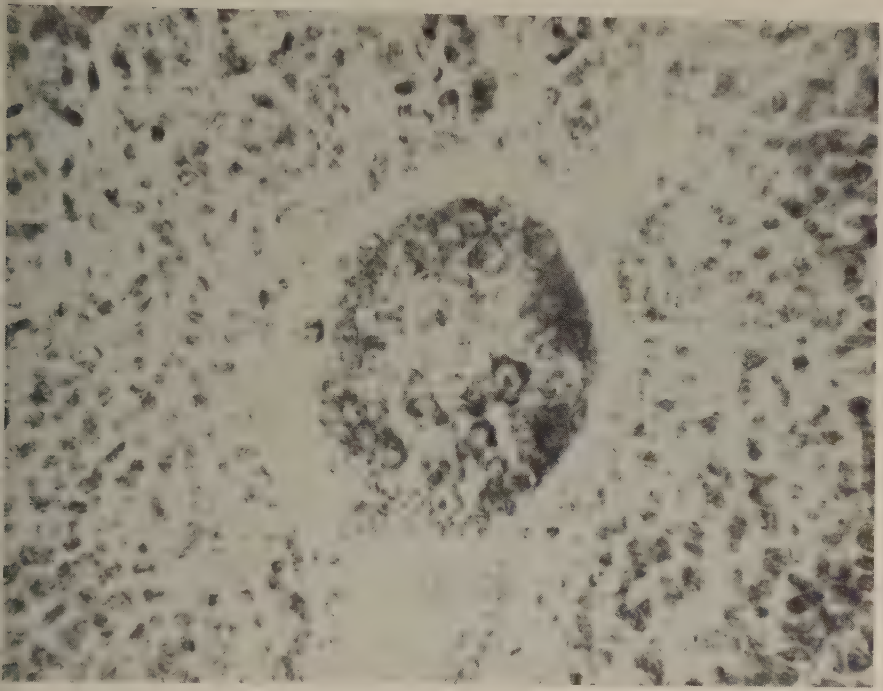

FIGURE 4. Spleen of 8-week-old germfree chicken: example of secondary nodule. ×250 (approx.).

The relationship (TABLE 3) of the development of the lymphoid tissue, particularly of the number of plasma cells, to the amount of gamma-globulin* in the serum of the germfree chickens has been described elsewhere.[1] The large differences between 4- and 8-week-old germfree and control animals in the number of plasma cells in the intestinal wall was not reflected in a similarly arge difference in the gamma-globulin content of the serum at that age. It would seem, therefore, that in control chickens not all the gamma-globulin produced in the intestinal mucosal lining reaches the serum; part of it may be excreted into the lumen of the intestines.[13, 14]

Rats. There was a fairly large difference in the number of plasma cells and secondary nodules in the spleens, mesenteric lymph nodes, and intestinal mucosal lining between control rats or "exgermfree" rats that had been exposed o normal environments for 4 weeks on the one hand and germfree or monocontaminated rats on the other (FIGURES 5 and 6). The rats that had been xgermfree for a period of 1 to 2 weeks showed interesting pictures of newly developing small secondary nodules in the mesenteric lymph nodes and of many aggregates of immature plasma cells in the spleen.

Glimstedt[15] and Miyakawa *et al.*[16] have stated that secondary nodules do not

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* Gamma-globulin was determined with the aid of a microelectrophoresis apparatus.

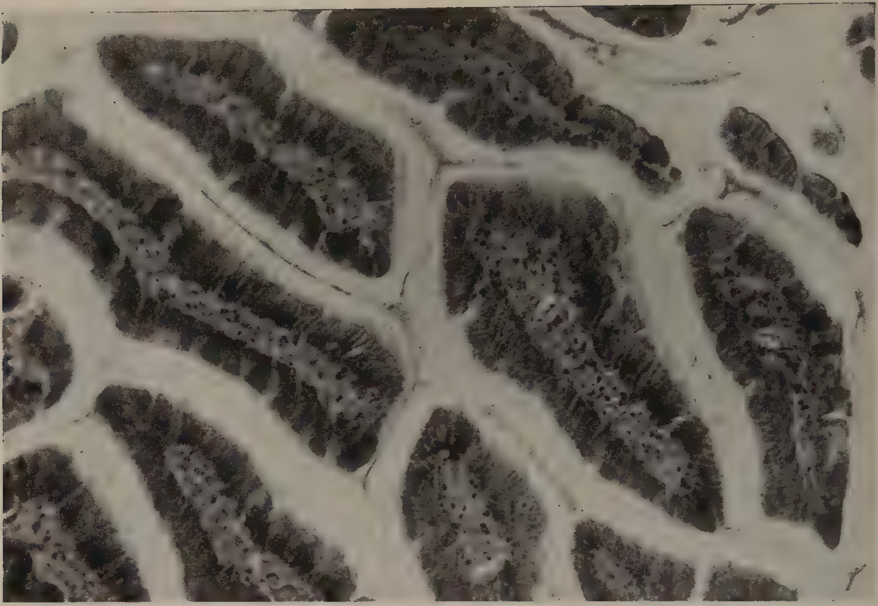

FIGURE 5. Ileum of a monocontaminated rat that had been exposed to normal environments for only 4 hours before being sacrificed: very few plasma cells in the stroma of the intestinal villi. ×100 (approx.).

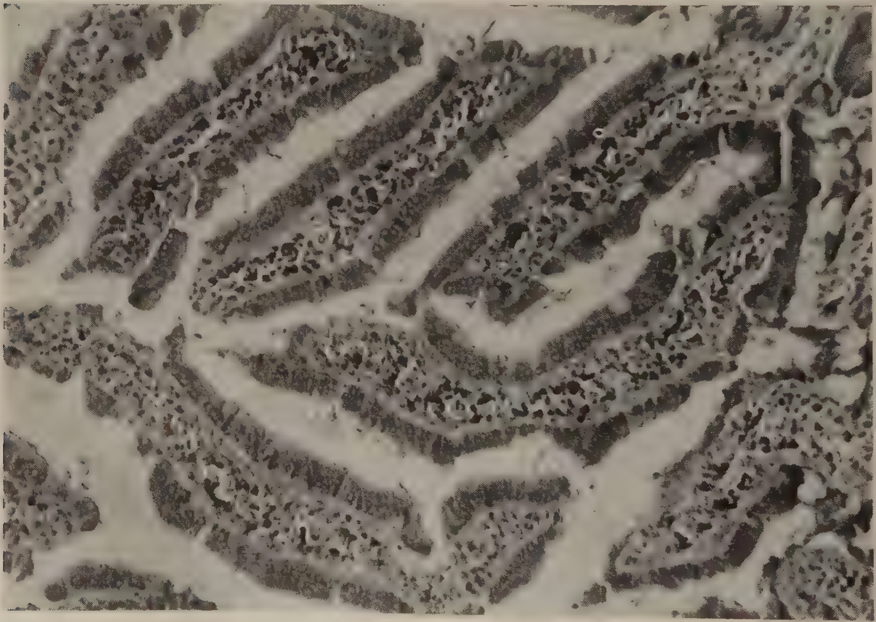

FIGURE 6. Ileum of a monocontaminated rat that had been exposed to normal environments for 4 weeks before being sacrificed: numerous plasma cells in the stroma of the intestinal villi. ×100 (approx.).

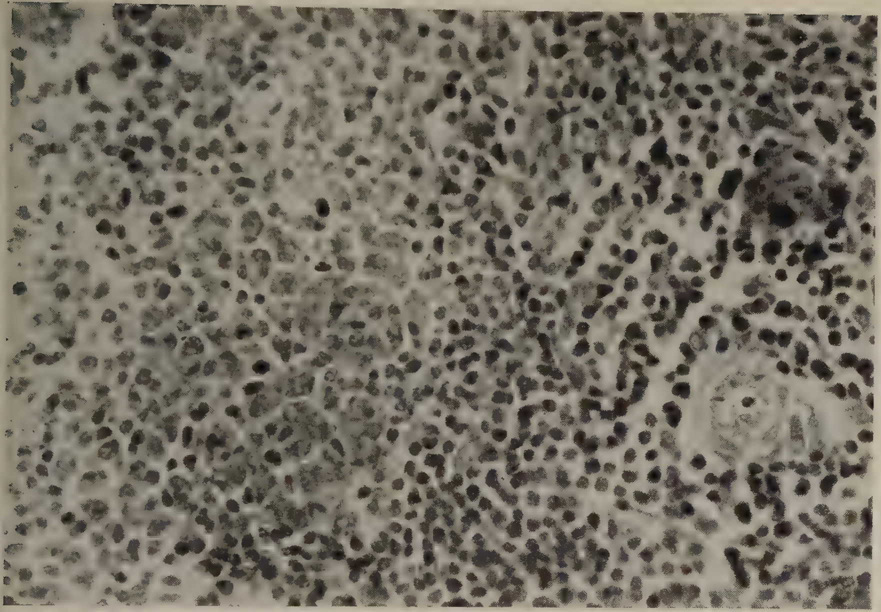

Figure 7. Spleen of a germfree rat: on the right is a fairly large reaction center, showing many mitoses. ×250 (approx.).

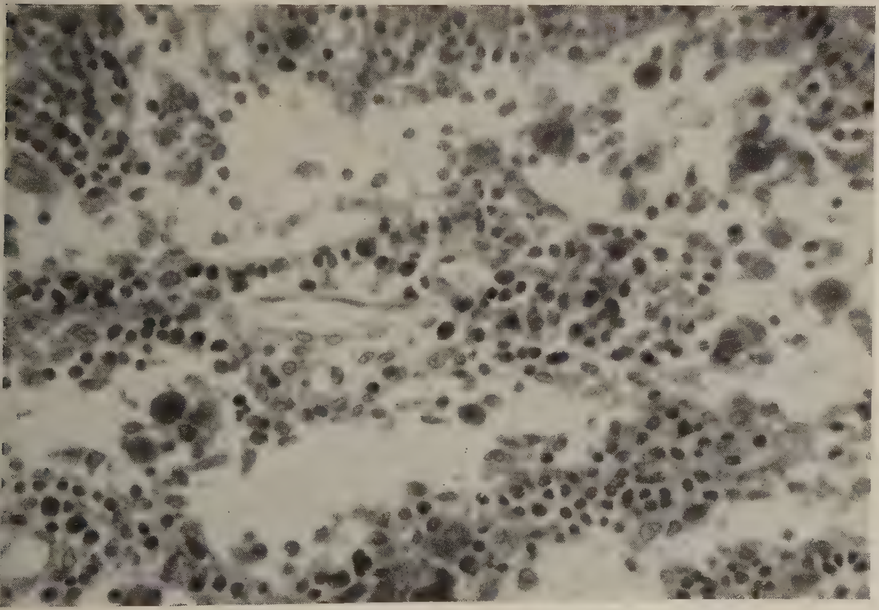

Figure 8. Mesenteric lymph node of a germfree rat: the medullary cords show a fair number of plasma cells and many macrophages containing PAS-positive material. ×250 (approx.).

occur in germfree guinea pigs. No such outstanding difference, however, was observed in these studies on rats. FIGURE 7 shows that secondary nodules can occur in the spleens of germfree rats. They were also observed occasionally in the lymphoid tissues of the appendixes and in the mesenteric lymph nodes of germfree rats. Plasma cells were found in most of the lymphoid organs of the germfree rats, but usually only in small numbers. FIGURE 8 shows a photograph of the medullary cords of a mesenteric lymph node of a germfree rat in which quite a few plasma cells were present.

From these results it is not possible to conclude whether the presence of plasma cells and of gamma-globulin in germfree animals is still a reflection either of antibody formation or of the formation of a "normal" gamma-globulin without antibody function.

Contact with antigenic materials such as food constituents, dead bacteria, dust constituents, viruses, or even autoantigens cannot be excluded. In other words, the germfree animals are only quantitatively, but not qualitatively, different from control animals in this respect.

It has been shown by Wagner[17] that some antibodies, such as bacterial agglutinins and heteroagglutinins, are formed in germfree animals. Furthermore, it was found[1, 17] that germfree animals are normally capable of forming antibodies and of reacting to multiple antigen injections with the formation of many plasma cells and secondary nodules in their organs.

Acknowledgments

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SOME HISTOLOGICAL AND FUNCTIONAL ASPECTS OF LYMPHOID TISSUE IN GERMFREE ANIMALS

II. STUDIES ON PHAGOCYTOSIS *IN VIVO*

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Introduction

Studies on phagocytosis in germfree animals have been concerned mainly with the phagocytic activity of blood leukocytes for bacteria *in vitro*. Differences in the phagocytic activity of circulating leukocytes from germfree and control rats were observed by J. P. Doll of the Lobund Institute, University of Notre Dame, Notre Dame, Ind., for certain, but not all, types of bacteria tested (personal communication). Wagner, elsewhere in this monograph, reports agglutinins for *Micrococcus epidermidis*, but not for *Escherichia coli* in germfree rats, and has confirmed similar observations in germfree mice (personal communication). The parallelism between the results obtained by these investigators indicates that the observed differences in phagocytic activity between germfree and control rats probably depended on the presence or absence of antibodies for the bacteria tested.

A few unpublished studies about the reticuloendothelial system of germfree animals *in vivo* have been made by C. Hyman of the University of California, Los Angeles, Cal. (unpublished data) and by H. A. Gordon of the Lobund Institute. These investigators have found that the Kupffer cells of the liver in germfree animals were quite capable of taking up substances such as Evans blue and Thorotrast.* Biozzi *et al.*[1] have shown that, in normal animals, the rate of phagocytosis for a standardized homogeneous carbon suspension of 250 Å particle size is dependent on the dose of the intravenously injected carbon. The rate of phagocytosis is expressed with the aid of a phagocytic index, K. This factor K is related to the concentration of carbon in the blood at various times after the carbon injection according to the equation $C_t = C_o^{-kt}$ and therefore can be calculated graphically. If the injected dose of carbon is kept constant, K is a measure of the phagocytic activity of the reticuloendothelial system (RES). K depends on the combined relative weights of liver and spleen according to the equation $\alpha =$ (body weight/weight liver and spleen)$\sqrt[3]{K}$. This factor α is a measure for the phagocytic activity of the RES as corrected for organ weight and has been found to vary little among different control mice for a given dose of carbon.

The clearance of heat-killed P^{32}-labeled bacteria from the blood stream, however, has been found to be relatively independent of the injected dose of bacteria. It is more subject to the influence of serum factors of which specific antibodies are the most important and act as a limiting factor.[2]

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* Thorotrast, produced by Testagar & Co., Inc., Detroit, Mich., is a 25 per cent suspension of thorium dioxide.

As a logical extension of the studies on germfree animals cited above, it was decided to examine *in vivo* both the rates of clearance for carbon particles and for heat-killed, P^{32}-labeled bacteria from the blood stream in germfree and control mice.

Materials and Methods

Animals. The germfree animals were housed in sealed rearing units as described by Reyniers *et al.*[3, 4] The germfree mice used in these experiments had been born naturally from germfree mice within the units and descended through several generations from cesarean-born hand-fed germfree Swiss Webster mice. Male and female mice approximately 40 days old, and weighing 20 to 30 gm. were used. The methods for testing their bacteriological sterility were the same as those in routine use at the Lobund Institute.

The germfree animals on which the clearance studies were performed were removed from the units one-half hour to two hours before use.

Control Swiss Webster mice were raised on the same autoclaved diet (Lobund Institute diet 356) as the germfree mice. Per 100 gm., this diet contains 20 gm. Labco casein; 58 gm. rice flour; 5 gm. cellophane spangles; 5 gm. salts; 5 gm. corn oil; 5 gm. yeast extract; 2 gm. Armour liver powder; and a mixture of vitamins.

Carbon clearance. The phagocytic activity of the RES for a homogeneous carbon suspension* was measured as previously described by Biozzi *et al.*[1] The mice were injected intravenously with 16 mg. carbon per 100 gm. body weight, and at various times after the injection (intervals of 2 to 5 min.) blood samples were obtained from the retro-orbital venous plexus with a calibrated (0.025 ml.) pipette previously rinsed with heparin. The blood was lysed in 2 ml. 0.1 per cent Na_2CO_3, and the amount of carbon determined in it photometrically at a wave length of 675 $m\mu$. The phagocytic indices, K, were calculated from the equation $K = (\log C_1 - \log C_2)/(t_2 - t_1)$ (FIGURE 1). The animals were sacrificed when most of the carbon had been cleared from the blood. The spleens and livers were weighed, the ratios body weight/weight liver plus spleen were calculated, and the corrected indices, α, obtained from the equation cited in the introduction.

Immunization. Five germfree and 5 control mice were immunized by an intraperitoneal injection of 3×10^8 heat-killed *Escherichia coli* 8 days before clearance studies with that pathogen were performed. In previous studies on normal mice[2] it had been shown that such an immunization induces an enormous enhancement of the clearance rates for these bacteria. Since it was also shown previously that autoclaving of the bacteria did not interfere with immunizing capacity in normal mice, the *E. coli* used for immunization within the germfree units were autoclaved in sealed ampules before they were introduced.

Bacterial clearance. *E. coli* (strain 0111: B_4, Difco) and *Staphylococcus aureus*

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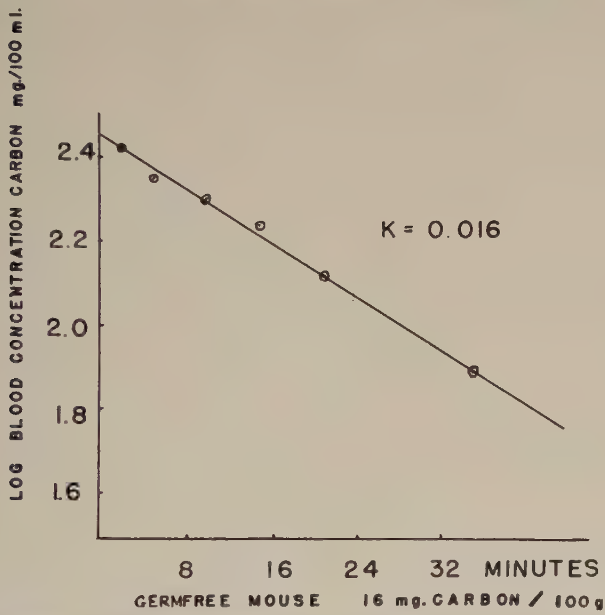
* Obtained from Gunther Wagner, Hanover, Germany.



IGURE 1. Example of a blood clearance curve for carbon obtained in a germfree mouse.

(Giorgio strain) were grown on a medium* containing as its phosphate source P^{32}-labeled phosphate (20 mc. $P^{32}O_4$ per 1000 ml. medium). The organisms vere killed by exposure to 60° C. for 30 min., washed several times, and re-uspended in saline.

The mice were injected intravenously with 1 ml./100 gm. body weight of a uspension containing 4×10^9 P^{32}-labeled *E. coli*/ml. or with a corresponding lose of a labeled *Staph. aureus* suspension of the same optical density. Five ninutes before the injection of bacteria, the mice received an intravenous in-ection of 0.05 to 0.1 ml. heparin in order to prevent clumping of the bacteria nd their entrapment in the lung capillaries.

Blood samples were taken with a calibrated (0.025- to 0.05-ml.) pipette at egular intervals after the injection of bacteria. The blood samples were laced on a dish of filter paper of 2-cm. diameter, dried, and measured for adioactivity with the aid of a Tracerlab TG C^{14} flow counter. The logarithms f the measured radioactivity counts were plotted with respect to time, and a traight line was drawn that fitted the points best. The slope of this line was alculated according to the equation $K = (\log C_1 - \log C_2)/(t_2 - t_1)$.

The mice were sacrificed immediately after the last blood samples were taken, hen a known percentage of the labeled bacteria was still present in the blood. n some cases the liver, spleen, kidneys, and lungs were removed, weighed, and

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[*] The medium contained sodium citrate·$3H_2O$, 0.5 gm.; $Mg_2SO_4 \cdot 7H_2O$, 0.1 gm.; $(NH_4)_2SO_4$, gm.; glucose, 2 gm.; casamino acids, 10 gm.; and distilled water to make 1000 ml.

TABLE 1

BLOOD CLEARANCE OF 16 MG. CARBON PER 100 GM. BODY WEIGHT IN GERMFREE AND CONTROL MICE

Germfree mice Sex	K	Ratio of body weight to weight of liver and spleen	α
M	0.025	20.3	5.9
M	0.014	23	5.6
F	0.021	20.3	5.6
F	0.016	22.6	5.6
M	0.009	22.3	4.7
F	0.012	23.4	5.4
Mean value	0.016	22	5.5
Normal* control mice	0.023 ± 0.016	15	3.8 ± 0.4

* From Biozzi *et al.*[5]

TABLE 2

THE INDIVIDUAL K VALUES FOR THE BLOOD CLEARANCE OF *E. COLI* (8×10^8 CELLS) IN GERMFREE, IMMUNIZED GERMFREE, CONTROL, AND IMMUNIZED CONTROL MICE

	Germfree	Immunized GF	Control	Immunized C
	0.0046	0.083	0.0107	0.074
	0.0094	0.104	0.0104	0.073
	0.0076	0.100	0.0115	0.070
	0.0040	0.097	0.0103	0.095
	0.0094	0.130	0.0090	0.097
	0.0060			
	0.0060			
Mean value	0.0067	0.103	0.0104	0.082

analyzed for radioactivity after hydrolysis in 10 per cent NaOH. The percentage of the injected radioactivity recovered from each organ was calculated.

Results and Discussion

FIGURE 1 presents an example of a carbon-clearance curve obtained in a germfree mouse. The curve looks exactly like one of those usually obtained with control mice. TABLE 1 shows that the values for the phagocytic indices, *K*, in germfree animals did not differ significantly from those observed in control mice. It was found, however, that the combined relative weight of liver and spleen was significantly lower in germfree than in control mice, chiefly because of a difference in relative liver weight.* The corrected indices, α, are therefore higher in germfree than in control mice. A similar difference has been found by Stiffel *et al.*[6] between newborn and adult rats.

TABLE 2 and FIGURE 2 summarize the results of the clearance studies with

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* The liver weights per 20 gm. body weight in 9 control mice were 1180 ± 80 mg., and in 15 germfree mice they were 900 ± 130 mg.

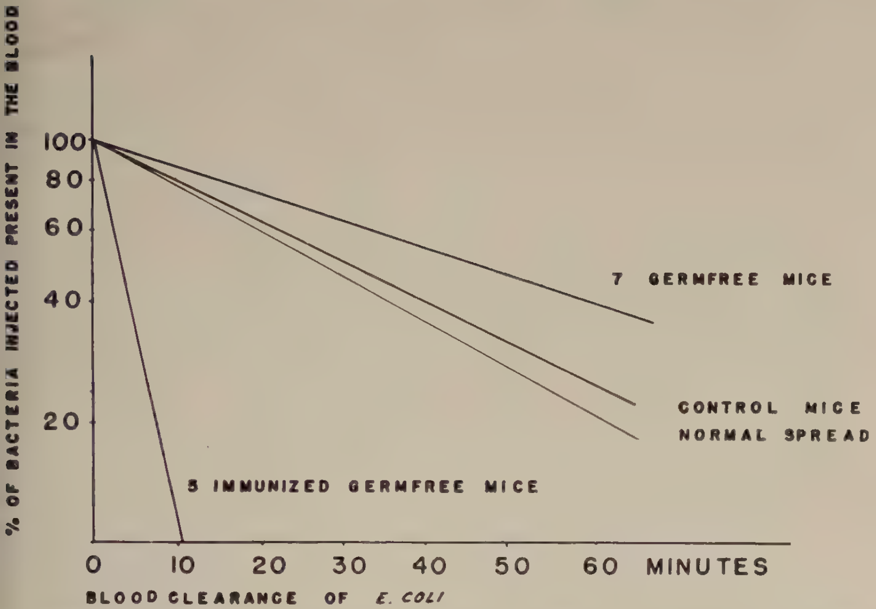

FIGURE 2. Blood clearance curves for heat-killed P^{32}-labeled *E. coli* drawn for the mean values of K obtained in groups of 7 germfree, 5 control, and 5 immunized germfree mice.

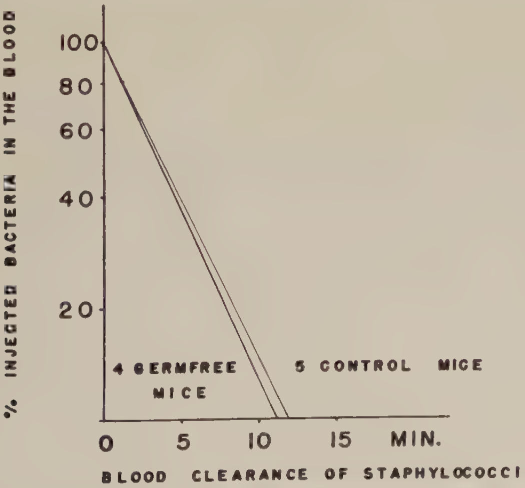

FIGURE 3. Blood clearance curves of heat-killed P^{32}-labeled *Staph. aureus* drawn for the mean values of K obtained in groups of 4 germfree and 5 control mice.

E. coli on germfree, immunized germfree, control, and immunized control mice. The control mice showed less variation in their clearance rates for these bacteria than did the germfree mice. It is apparent, however, that the clearance of *E. coli* in the germfree mice was slower than in control mice.

Immunization with *E. coli* 8 days before the clearance studies effected a similar enhancement of the clearance rates for *E. coli* in both groups.

The clearance rates for *Staph. aureus* were similar in germfree and control mice. *Staph. aureus* were removed much faster in both germfree and control mice than were *E. coli*; the mean value for the clearance rates in 4 germfree mice was 0.096 and, in 5 controls, it was 0.093 (FIGURE 3). Since the clearance

TABLE 3

DISTRIBUTION OF RADIOACTIVITY AMONG VARIOUS ORGANS AND BLOOD AFTER BLOOD CLEARANCE OF P^{32}-LABELED *E. COLI*

Percentage in	Control No. 1	Control No. 2	Control No. 3	Control No. 4	Average controls	Immunized control No. 5
Liver	27.5	27.0	18.0	27.0	25.0	75.0
Spleen	18.0	18.0	15.0	10.5	15.4	6.5
Lungs	5.5	6.4	3.1	4.7	4.9	8.0
Kidneys	1.8	1.6	1.4	1.7	1.6	2.0
Blood	20.0	22.5	22.0	30.0	23.8	4.0
Total recovered	72.8	75.5	59.5	73.9	70.7	95.5
Percentage in	Germfree No. 6	Germfree No. 7	Germfree No. 8	Germfree No. 9	Average germfree	Immunized germfree No. 10
Liver	22.0	20.5	17.2	26.0	21.4	74.0
Spleen	13.0	5.6	20.5	20.0	14.8	8.0
Lungs	7.5	6.6	6.5	7.5	7.0	1.5
Kidneys	2.0	5.0	2.0	1.0	2.5	1.9
Blood	31.0	45.0	26.5	18.5	30.2	6
Total recovered	75.5	82.7	72.7	73	75.9	91.4

TABLE 4

DISTRIBUTION OF RADIOACTIVITY AMONG VARIOUS ORGANS AND BLOOD AFTER BLOOD CLEARANCE OF P^{32}-LABELED *STAPH. AUREUS*

Percentage in	Control No. 1	Control No. 2	Control No. 3	Average controls
Liver	64.0	79.0	80.0	74.0
Spleen	2.3	5.3	9.0	5.5
Lungs	2.4	0.3	0.4	1.0
Kidneys	0.3	0.3	0.4	0.3
Blood	6.0	4.5	3.0	4.5
Total recovered	75.0	89.4	92.8	85.3
Percentage in	Germfree No. 4	Germfree No. 5		Average germfree
Liver	62.0	65.0		63.5
Spleen	9.0	6.0		7.5
Lungs	7.5	1.7		4.5
Kidneys	1.0	0.3		0.7
Blood	6.0	6.5		6.2
Total recovered	85.5	79.5		82.4

ates for *Staph. aureus* were already so high, an effect of previous immunization ith these bacteria could not be shown.

Tables 3 and 4 summarize the results of the studies on the recovery of radioctivity in organs and blood after most of the bacteria had been cleared from he blood. No differences between germfree and control mice were observed 1 this respect. Table 3 shows that in immunized animals a much higher perentage of the injected bacteria go to the liver than in nonimmunized mice. It as found (table 4) that the organ distribution of *Staph. aureus* in nonimmuized mice is similar to that of *E. coli* in mice previously immunized with the itter pathogen.

Since it has been shown by Wagner elsewhere in these pages that antibodies gainst certain bacteria, particularly against staphylococci, are found in germee animals, these results are in accordance with other studies[2] indicating hat antibodies are of major importance in determining the rate of clearance f bacteria from the blood stream.

These histological and serologic observations (see also Wostmann, elsewhere 1 this publication) and studies on phagocytosis contribute to the rather incomlete knowledge of the status of the defense mechanism in germfree animals. t appears that, although the animals are capable of responding very well, they sually show a lower development of their lymphoid tissue, lower serum gammalobulin levels and, in certain cases, lower phagocytic indices for bacteria.

However, more important qualitative differences in this respect may not beome apparent until it is possible to raise the animals free, not only from all emonstrable living bacteria, but also from dead bacteria and other antigenic ibstances.

Acknowledgments

The kindness of J. A. Reyniers, H. A. Gordon, M. Wagner, and B. S. Wostlann in providing the germfree animals used in these studies is acknowledged. Vithout the advice and assistance of these investigators the work reported in is paper could not have been done.

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SERUM PROTEINS IN GERMFREE VERTEBRATES*

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The germfree animal has been bred and reared in the absence of any demonstrable microbial form. Thus it seems logical to expect that those serum protein fractions that normally harbor antibacterial antibodies will show a certain deficiency in the germfree animal.

Circulating antibodies have been defined chiefly as gamma-globulins. Tiselius and Kabat[1] have shown that in rabbits practically all circulating antibodies against a number of antigens are found in the gamma fraction. Conclusive evidence now seems available to show that, unlike most other serum protein, gamma-globulin is formed outside the liver.[2, 3] The apparently unique position of the gamma-globulin causes certain investigators to regard this fraction as being composed solely of antibodies against a variety of known and unknown antigenic stimuli.[4, 5]

However, the work of Olberg[6] with mice has associated the alpha-globulin fraction with antibody formation against *Trypanosoma brucei*. Leland and his co-workers[7] found an increase in beta-globulin levels in rats upon infection with *Nippostrongylus muris*. To my knowledge, no special serum fraction has been associated with antibacterial antibodies in rats and mice.

Circulating antibodies occupy a key position in the defense system of the host. Their identification in the serum protein fractions and the subsequent isolation of these fractions will enable further study of such matter as the sites of formation of the various kinds of antibodies and their specific functions. The serum of the germfree animal may be expected to suggest, by certain deficits, those serum protein fractions in which antibacterial antibodies are apparently missing, and thereby indicate the fractions in which those antibodies are normally present. As a next step, controlled antigenic stimulus of the germfree animal will bring about responses that one may expect to find reflected in the serum protein pattern, where they can be followed as to time and magnitude. Thus far, the electrophoretic pattern of germfree rats and chickens has been thoroughly explored. Somewhat more limited material on germfree Swiss mice is available. Our data on germfree guinea pigs and rabbits are still limited by the fact that valuable adult material could not be risked to obtain the necessary serum. At present the foremost duty of adult germfree rabbits and guinea pigs is propagation of their species. For this reason the data on these animals are from material that had to be sacrificed because of very recently detected contamination. In rats it was repeatedly found that, for the first week after the detection of bacterial contamination, no influence on the serum protein pattern could be detected. Our data on guinea pigs and rabbits must therefore be regarded in the light of these facts.

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* The work reported in this paper was supported in part by Contract NONR-1623(04) between the Office of Naval Research, Department of the Navy, Washington, D. C., the Gustavus and Louise Pfeiffer Research Foundation, New York, N. Y., and the University of Notre Dame.

Methods. The analysis of the serum was done using the Antweiler micro-ectrophoresis technique. A barbital buffer, $\mu = 0.12$ and $pH = 8.6$, was sed throughout. It should be mentioned that, compared with the usual iselius technique, the Antweiler technique results in somewhat higher albumin nd somewhat lower beta-globulin values.[8]

Results

Chickens.[9, 10] A typical serum electrophoretic pattern of the 3-month-old ermfree chicken is shown in FIGURE 1. It demonstrates that the principal ifference between normal and germfree stock is in the gamma-globulin fraction. ABLE 1 shows that the gamma-globulin values in the germfree birds remain w as the animal matures, while the normal stock chicken shows a steady in-ease with time until about 30 per cent of the total serum protein is taken up y the gamma-globulins. Parallel to this rise in gamma-globulin with age oes a rise in various antibody titers (coliforms and micrococci). All this

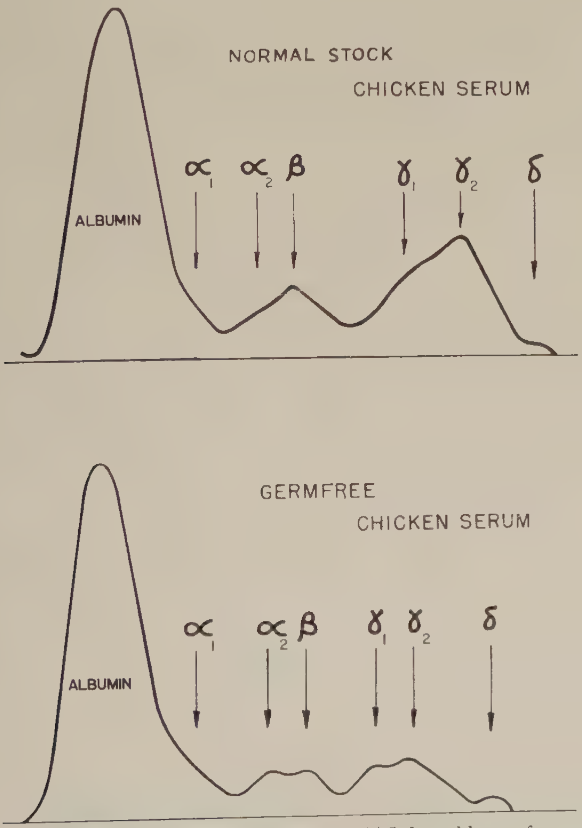


FIGURE 1. Electrophoretic patterns of the serum of 75-day-old germfree and normal stock hickens.

TABLE 1

SERUM PROTEIN FRACTIONS IN GERMFREE (GF) AND NORMAL STOCK (NS) CHICKENS (SEXES MIXED)

S.D.M Values Given

Age (days)	Units	Albumin*		Alpha-1-globulin		Alpha-2- plus beta-globulin		Gamma-globulin	
		GF	NS	GF	NS	GF	NS	GF	NS
35	Mg. per cent	2775 (8) ±94	2315 (9) ±89	102 ±8	95 ±24	353 ±31	332 ±21	331 ±21	443 ±23
75	Mg. per cent	2673 (5) ±79	2623 (11) ±115	170 ±31	108 ±19	306 ±27	341 ±25	321 ±43	800 ±55
125	Mg. per cent	2753† (4) ±141	2450 (10) ±72	160† ±50	106 ±10	442† ±32	506 ±31	397† ±20	1171 ±77

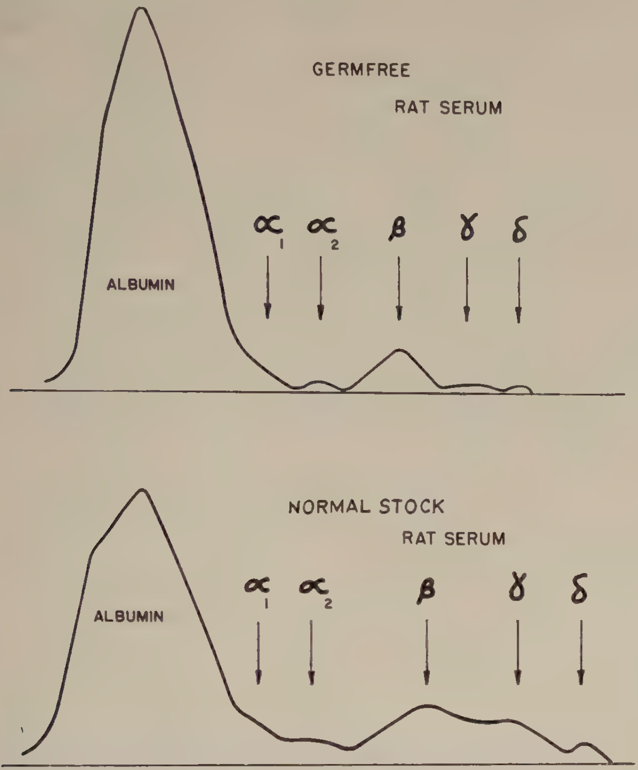
* The number of experimental animals is indicated in parentheses.

† Chickens became contaminated with a pure culture of *Micrococcus* sp. 1 to 6 days before sacrifice.

seems to prove that the deficit in gamma-globulin seen in the case of the germfree chicken is indeed caused by a lack of antibacterial antibody.

However, even in the germfree bird a certain small amount of gamma-globulin is always present. The fact that this amount does not show any substantial increase with age apparently indicates that most of this fraction is not formed as a result of any strong, persistent antigenic stimulus. Here the confusing problem of the so-called "natural antibody" seems to present itself. Wagner[11] and Reyniers[12] have found agglutinating antibodies against rabbit erythrocytes in the serum of germfree chickens at 30 days of age or older. Springer and his co-workers, on the other hand, find antibody against human group B blood substance absent in germfree chickens; however, it is present in normal stock animals. These antibodies seem to appear when the germfree birds are stimulated by contamination with a strain of *Escherichia coli*.[13] A certain antigenicity of the diet due to residues of autoclaved bacteria and other antigenic material is another possibility. This is indicated by the positive agglutination with certain bacterial suspensions, for example, *Micrococcus epidermidis* in germfree chickens older than 200 days. As a whole, the nature of this "germfree" gamma-globulin is little understood.

Some results obtained with the feeding of antibiotics to normal stock chickens may be mentioned briefly here. The administration from birth of 50 mg. of procaine penicillin G per kilogram of diet gives rise to many germfreelike characteristics, expressed by lower weight and less lymphoid tissue content of those organs normally in contact with an abundant flora. The serum protein pattern in this case seems hardly affected by the administration of the antibiotic. The data show that only for animals more than 3 months of age the level of gamma-globulin in the treated birds falls below that reached by the normal stock animal of the same age. At the age of 4 months the average level has barely increased above the value of approximately 800 mg. per cent seen at 75 days of age (actual average 877 ± 56 mg. per cent against 1171 ± 77 mg. per cent for the untreated animals).



IGURE 2. Electrophoretic patterns of the serum of 90-day-old germfree and normal k rats.

TABLE 2

UM PROTEIN FRACTIONS OF GERMFREE AND NORMAL STOCK 90-DAY-OLD MALE RATS*
S.D.M. Values Given

	Germfree (10 rats) Mg. per cent	Normal stock (9 rats) Mg. per cent	P
al serum protein	5833 ± 163	5961 ± 141	0.78
umin	5098 ± 179	4272 ± 136	<0.01
ha-1-globulin	103 ± 14	135 ± 14	0.19
ha-2-globulin	69 ± 13	271 ± 41	<0.01
a-globulin	388 ± 48	804 ± 69	<0.01
nma-globulin	175 ± 28	479 ± 53	<0.01

Lobund strain.

ats. FIGURE 2 shows representative results obtained from 90-day-old mfree and normal stock albino rats. TABLE 2 shows that significantly er values in germfree rats are found in the alpha-2, beta, and gamma fracs. Apparently this deficit is compensated by a higher albumin level, bringing the total protein concentration of the serum (and thereby the colloid-osmotic value) to a value comparable to that seen in the normal stock animal.

It may be mentioned here that, if the germfree rat is removed from its germfree environment and seeded with a "normal" cecal flora, the fractions alpha-2, beta, and gamma increase, one after the other, in this sequence. Two weeks after this contamination the alpha-2 level has covered roughly two thirds of the way toward normal values, and the beta level has just started to increase, while the gamma value is still at the germfree level.

As in the chicken, the germfree Lobund rat shows essentially all the serum-protein fractions found in the electrophoresis pattern of the normal stock animal.

Swiss mouse. In a preliminary survey of the germfree Swiss mouse, the situation seems to resemble that of the rat in many respects (FIGURE 3). Once again all serum protein fractions are present in germfree mice, while numerical

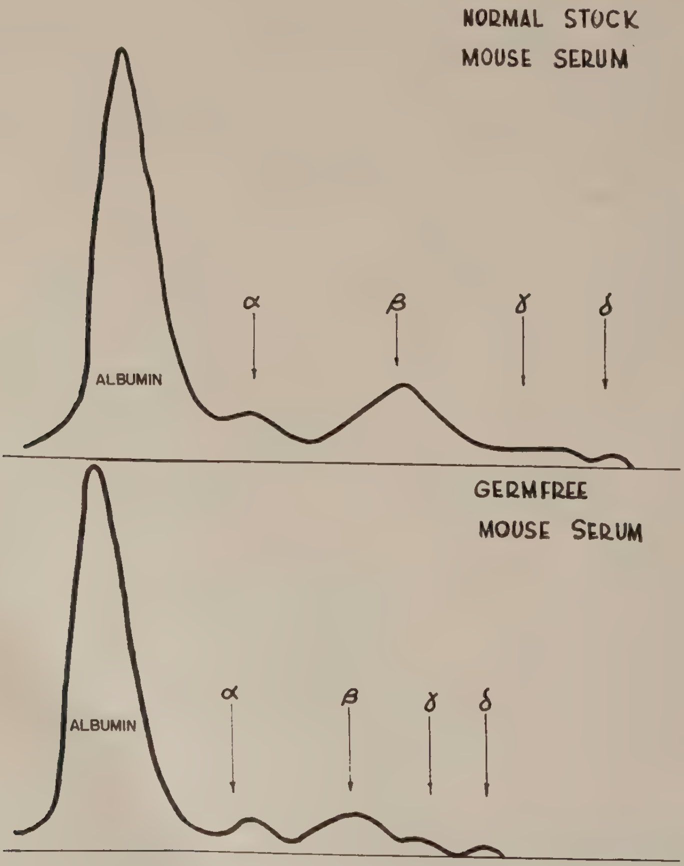
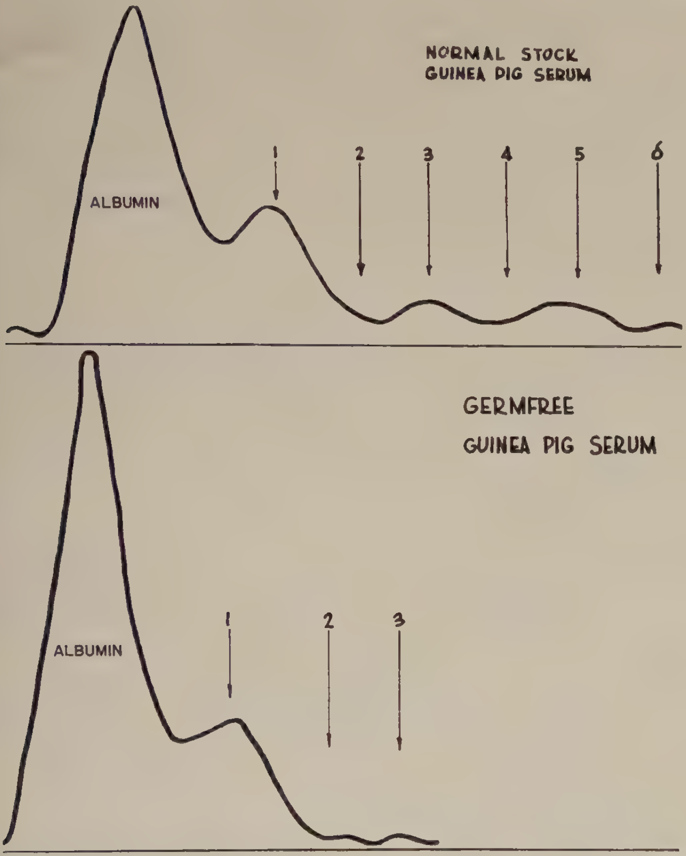


FIGURE 3. Electrophoretic patterns of the serum of 60-day-old germfree and normal stock Swiss mice.



IGURE 4. Electrophoretic patterns of the serum of adult "germfree" (see text) and nor- stock guinea pigs.

ı show that beta and gamma fractions are definitely lower. Data from a er number of animals may prove the same for the alpha-2 fraction.

uinea pig. Data are available on four 1-year-old guinea pigs that it was ssary to sacrifice because of contamination with a strain of micrococci. animals were sacrificed a few days after the contamination could possibly e occurred. FIGURE 4 shows the interesting fact that in this case at least of the slower-moving (gamma-) globulin fractions are missing entirely in exgermfree animals. Fraction 3, which could be designated as the beta ion because of its mobility, was present in most instances, although at a h lower level than in normal stock animals.

ne fact should be stressed that this is the only case we encountered in which ain normally occurring serum protein fractions were completely missing in nimal with a germfree history.

ıbbits. As I have mentioned in an earlier presentation in this monograph, ıfree rabbits are now available, although the present animals are not yet oletely satisfactory. The older ones do not appear to be healthy at times, and I suspect that the diets are not entirely adequate. The rabbit, as well as the chicken, is known as an excellent producer of antibody. Consequently, it was surprising that in the serum pattern of two adult exgermfree rabbits (taken directly after the contamination became known) no obvious differences could be found from the normal stock animal. In this case there may be some doubt as to the validity of these results because of a slow-growing *Micrococcus* sp. that was present no more than five days before these serum samples were obtained. However, in all other species observed in this laboratory, antibody response as indicated by the serum electrophoresis pattern was never as rapid.

Discussion

The data presented suggest, among other things, that the responses in rats and mice against *Trypanosoma* and *Nippostrongylus* infections, which indicated the occurrence of circulation antibody in the alpha and beta fractions, seem to be paralleled in the case of antibacterial antibodies. Furthermore, the gamma fraction also reacts upon bacterial invasion. The slowness of the reaction (lag time at least two weeks) may be the reason that this fraction escaped notice in earlier work.

Another point is the presence or absence of certain globulin fractions in the germfree animal. We find that a certain content of gamma-globulins is present in the serum of germfree rats, mice, chickens and, in all probability, rabbits. On the other hand, a definite lack of certain (gamma) fractions is seen in the germfree guinea pig. It is conceivable that this absence of certain fractions can be related to the fact that these guinea pigs were first-generation animals obtained by cesarean section and brought up without the normal contact with a mother animal. The germfree rats and mice were at least of the fifth generation.

In general, these data show that valuable information can be obtained from the study of the serum electrophoresis patterns of germfree and exgermfree animals. The total absence of certain fractions in germfree animals is especially intriguing. In this respect it seems of the utmost importance to rear germfree animals with an absolute minimum of antigenic stimuli from such factors as dietary substances and dead bacteria in order to bring out as clearly as possible the effects already mentioned.

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SEROLOGIC ASPECTS OF GERMFREE LIFE*

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Since the latter part of the Nineteenth Century numerous investigators have concerned themselves with the study of so-called natural antibodies. These antibodies have been called natural because they can be demonstrated in the sera of normal animals that have not been subjected to any injection or treatment with a specific antigenic material by the investigator. In the case of the natural antibacterial antibodies against specific pathogens, these antibodies can frequently be demonstrated in animals not known to have suffered any clinical symptoms of the disease. In many cases there has been no evidence of any contact between pathogen and host, so that even subclinical infection could not always explain the presence of antibacterial antibody for a specific organism. However, an accumulation of knowledge regarding the antigenic components of plants, animals, and microorganisms has shown that they may all share common or closely related antigenic substances. It is therefore no longer necessary to assume that each antibody entering into *in vitro* serologic reactions with a particular organism has been specifically produced in response to that organism. Wiener[1] has presented a similar argument for the origin of the natural human isoantibodies anti-A and anti-B. He argues that, while production of the human blood group substances A and B is controlled genetically from within, the stimulus for the production of the reciprocal isohemagglutinins arises from without through exposure to microorganisms that contain substances similar to human blood groups.

Historically, studies on the occurrence of natural antibodies have been repeated as improvements in animal care, isolation, sanitation, and nutrition have been made. The availability of animals reared free of known viable microbial life poses the question of whether natural antibodies make their appearance under conditions of germfree life. However, it should be kept in mind that the sterile diets used in this work are not free of antigens. This is a refinement that has not yet been attempted.

Observations were made on the occurrence of heterohemagglutinins and antibacterial agglutinins in the sera of chickens and rats under the following four conditions: (1) germfree animals undergoing no specific antigenic treatment (the normal germfree animal); (2) antibody response of germfree animals to parenteral injection of nonviable antigens; (3) antibody response of germfree animals to dead bacteria purposely incorporated in the diet; and (4) antibody response of "germfree" animals to monocontamination with pure cultures of viable bacteria.

Limited observations also were made on properdin and complement titers in germfree rats.

Since this monograph on germfree vertebrates includes consideration of the

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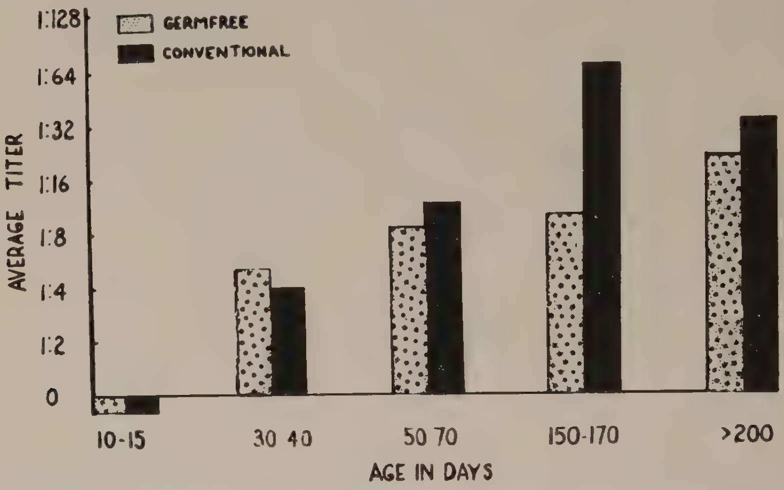

FIGURE 1. Heterohemagglutinins in normal germfree and normal conventional chicken serum for rabbit erythrocytes.

present status of its field of interest, it seems permissible in certain instances to present data as simple observations where explanations of results are not clearly understood. This allows for speculation and points out avenues that are still unexplored.

Most of the work that has been done on chickens is presented in review and has been published elsewhere.[2, 3]

Heterohemagglutinins in Normal Germfree Chickens

Hemagglutinins for rabbit erythrocytes in normal germfree and normal conventional White Wyandotte Bantam chicken serum are shown in FIGURE 1. No hemagglutinins were observed in young germfree or conventional chicks. However, at 30 days of age and older, agglutinins appeared in both categories. The same general observation was made with rat erythrocytes titrated against the normal germfree and conventional chicken sera. However, titrations with human, horse, and sheep erythrocytes yielded negative titers in both serum categories for all ages. These negative findings are contrary to the observations of Bailey,[4] who found low-titer hemagglutinins against these latter species erythrocytes in conventional chickens.

Springer *et al.*[5] recently reported that conventional Leghorn chickens have hemagglutinins for human type B erythrocytes, but that germfree chicks do not. Contamination of germfree chicks with *Escherichia coli* O_{86} (a strain possessing human blood group B activity) resulted in the appearance of agglutinins for human group B erythrocytes. The presence of so-called "natural" antihuman B hemagglutinins in conventional chicken serum may therefore be of immunogenic rather than natural origin. The presence of antirabbit and antirat hemagglutinins in our germfree chickens may represent naturally occurring hemagglutinins but, more probably, they also have been produced in response to some as-yet-unrecognized antigenic component of the autoclaved diets used.

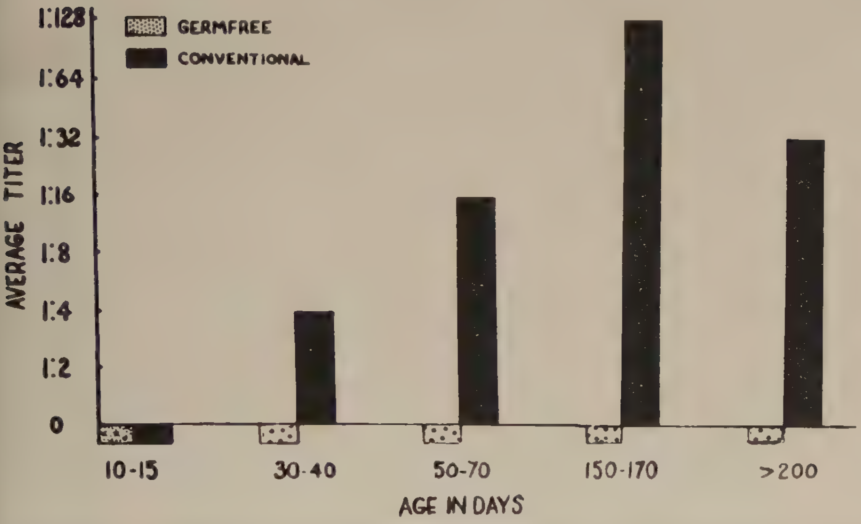

FIGURE 2. Agglutinins in normal germfree and normal conventional chicken serum for *Paracolobactrum aerogenoides* (fecal strain).

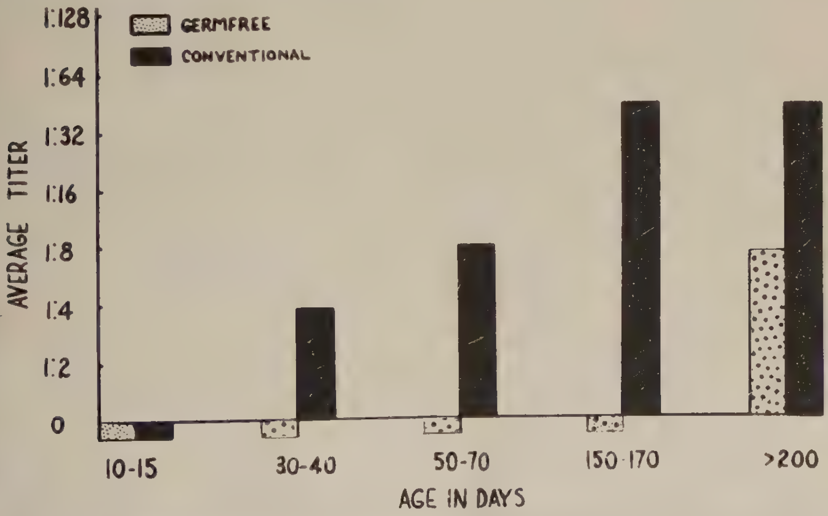

FIGURE 3. Agglutinins in normal germfree and normal conventional chicken serum for *Micrococcus epidermidis* (diet strain).

Antibacterial Agglutinins in Normal Germfree Chickens

Examples of antibacterial agglutinins in normal germfree and normal conventional chicken sera are shown in FIGURES 2 and 3. *Paracolobactrum aerogenoides*, one of the more predominant bacteria found in the feces of conventional chickens, was used as a test antigen (FIGURE 2). Conventional chicken sera gave negative titers in the youngest age group, but chicks 30 days of age and older showed positive titers. Germfree chicken sera showed negative titers at all ages tested.

The same sera titrated with *Micrococcus epidermidis* suspension showed positive titers in sera from conventional chicks 30 days of age or older (FIGURE 3). The germfree sera were negative in birds up to 150 to 170 days of age but, contrary to the totally negative results of *P. aerogenoides*, the >200-day age group showed positive titers.

Obviously, the germfree chickens did not harbor viable *P. aerogenoides* or *M. epidermidis*. However, the diets used for the germfree chicks were demonstrated to contain *M. epidermidis* as one of the most predominant bacterial forms prior to autoclaving, while *P. aerogenoides* or other coliforms could not be isolated. The autoclaved diet apparently contained enough dead *M. epidermidis* to stimulate a response in the germfree host by a process similar to immunization with killed oral vaccines. The delayed response was probably a function of low dosage. According to the bacteriological findings in the diet prior to sterilization, there was no stimulus or insufficient stimulus for the production of anti-*P. aerogenoides* agglutinins.

Antibacterial Agglutinin Response in Germfree Chickens Fed Killed Bacteria Purposely Incorporated in the Diet

In view of the previous observations and discussion on antibacterial agglutinins, an experiment summarized in FIGURE 4 was set up to check the ability of dead bacteria in the diet to stimulate agglutinins via the oral route. *P. aerogenoides* was grown in mass culture, washed, and concentrated to a thick bacterial paste. The paste was added to the diet at a 1 per cent level, autoclaved, and fed to 4½-week-old germfree chicks for a period of 3 additional weeks. Conventional chicks on the basic diet or diet plus bacteria showed

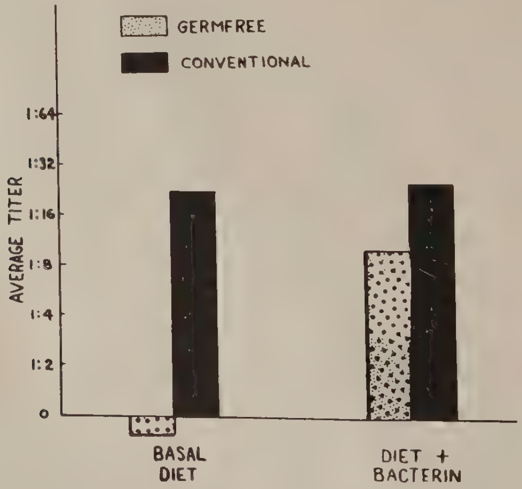

FIGURE 4. Agglutinin response in germfree chickens fed 1 per cent killed *Paracolobactrum aerogenoides* in the diet.

equivalent titers, indicating that the dead bacteria in the diet had no additional effect when superimposed over the normal contact with the viable coliforms in the intestinal tract. However, under germfree conditions, chicks on the basic diet remained negative, while the bacterin-containing diet stimulated agglutinin production.

Consequently it appears that at least certain so-called natural heterohemagglutinins and natural antibacterial agglutinins observable in conventional animals probably arise immunogenically.

Antibody Production in Germfree Chickens Injected Parenterally with Killed Salmonella pullorum or Beef Serum

Gordon,[3, 6] in describing germfree animal life, has indicated that certain structures associated with defense mechanisms, such as lymph nodes and ymphoid tissue, show less activation under germfree conditions, particularly n areas normally in direct contact with bacteria under conventional conditions. Other workers have also indicated only partial activation of defense systems in germfree chickens: namely, Thorbecke *et al.*[7], fewer plasma cells, lymphoid tissues, and gamma-globulins; Wostmann,[8] lower gamma-globulins; and Wagner,[2, 3] fewer antibodies.

Figure 5 is presented to indicate that under the conditions of this experiment the germfree chicken is not handicapped in its ability to produce antibody in response to parenteral antigenic stimulation.[3] Nontreated germfree and conventional chicks showed no titers to the *Salmonella pullorum* and beef serum antigens tested. Germfree and conventional chicks responded equally well to parenteral injection with the respective antigens used.

In summarizing the work with chickens, it appears that the so-called natural heterohemagglutinins and antibacterial agglutinins observable in conventional

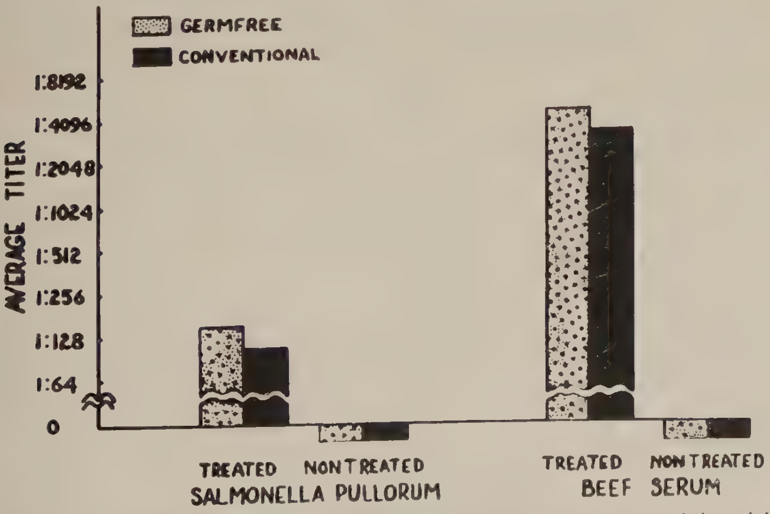

Figure 5. Antibody production in germfree and conventional chickens injected intravenously with *Salmonella pullorum* or beef serum.

chickens probably arise immunogenically rather than spontaneously. However, the spontaneous origin of some antibodies of this type cannot be entirely negated at present.

Observations similar to those reported for chickens have also been made in regard to the germfree rat.

Heterohemagglutinin Titers in Normal Germfree Rats

Heterohemagglutinins in normal germfree and normal conventional rat sera are shown in TABLE 1. In our experience, germfree and conventional rats up to the age of 250 days did not show heterohemagglutinins against a variety of species erythrocytes; conventional rats older than 300 days produced low titers. However, data for correspondingly older germfree rats are not yet available.

Antibacterial Agglutinins in Normal Germfree Rats

Antibacterial agglutinin titers in the normal sera of germfree and conventional rats are presented in TABLE 2. Ignoring for the moment the data

TABLE 1

HETEROHEMAGGLUTININ TITERS IN NORMAL SERA OF GERMFREE AND CONVENTIONAL RATS*

Antigen (RBC)	Germfree age 100 to 250 days	Conventional age	
		100 to 250 days	>300 days
Bovine	0 (1)	0 (2)	1:4 (3)
Human	0 (14)	0 (5)	1:2 (3)
Rabbit	0 (6)	0 (5)	
Sheep	0 (3)	0 (7)	1:4 (3)
Chicken	0 (7)	0 (7)	1:8 (3)
Horse	0 (7)	0 (7)	1:16 (3)

* Number of animals observed appears in parentheses.

TABLE 2

ANTIBACTERIAL AGGLUTININ TITERS IN NORMAL SERA OF GERMFREE AND CONVENTIONAL RATS

Antigen	Germfree age group (days)			Conventional age group (days)		
	<30	30 to 99	100 to 250	<30	30 to 99	100 to 250
Micrococcus epidermidis I*	0 (9)†	0 (4)	0 (23)	0 (9)	4–64 (4)	8–256 (23)
II*	0 (3)	32 64‡ (10)	32 64 (7)			
Escherichia coli	0 (11)	0 (8)	0 (23)	0 (9)	4 (3)	2–16 (29)
Streptococcus fecalis			0 (6)		2–4 (3)	2–256 (38)
Lactobacillus (heteroferm.)			0 (4)		0 (3)	8–16 (7)
Lactobacillus (homoferm.)			0 (13)	0 (8)	0 (1)	0 (7)

* I represents data obtained prior to January 1956; II represents data obtained thereafter.
† Number of animals observed appear in parentheses.
‡ Range of titers expressed as reciprocal of dilution.

under *Micrococcus epidermidis* II, sera from germfree rats as old as 250 days showed negative titers to the test antigens used. Sera from young conventional rats were also negative, but antibacterial agglutinins began to appear at adolescence. It is interesting to note that the appearance of agglutinins for heteroermentative lactobacilli was delayed when compared to *M. epidermidis*, *E. coli*, or *Streptococcus fecalis*. Since lactobacilli are generally considered to be rather weak immunogenically, the delayed appearance might be expected. It s also interesting to note that no agglutinins appeared for the homofermentative lactobacilli. The apparent absence of agglutinins for the homofermentative lactobacilli may be related to the observation that these forms apparently do not make up an appreciable part of the normal intestinal flora of conventional rats at the Lobund Institute. While lactobacilli have been demonstrated to constitute the most predominant entities of the intestinal flora in rats fed a high carbohydrate diet, these bacteria all appear to belong o the heterofermentative group. The same delayed appearance of agglutinins or heterofermentative lactobacilli and the absence of agglutinins for homoermentative types have also been observed in chickens.[3]

The results with *M. epidermidis* shown in TABLE 2 are presented in two parts. Part I presents data obtained prior to January 1956, while part II postdates hat time. All germfree rats tested before 1956 showed no agglutinins for *M. epidermidis*; all rats tested later showed positive agglutination. Positive iters were also observed in germfree mice during the later period. At present, here is no proved explanation for this change. The rats and mice all test ermfree; the dietary ingredients were obtained from the same sources; the iable *Micrococcus* count of the diet before autoclaving is approximately the ame as in earlier counts; and the test organism itself has not varied. The only hange coincident with the change in the agglutinin picture has been sterilization of the diet in pellet form rather than in its original granular form. While pelleting does not interfere with sterilization of the diet, it may possibly exert

TABLE 3

ANTIBACTERIAL AGGLUTININ TITERS IN NORMAL SERA OF GERMFREE, MONOCONTAMINATED,* AND CONVENTIONAL RATS
(AGE 100 TO 250 DAYS)

Antigen†	Germfree	Monocontaminated*	Conventional
treptococcus fecalis	0 (6)‡	64–2048§ (22)	2–256 (38)
treptococcus liquefaciens		1024 (4)	2–128 (8)
actobacillus (heterferm.)	0 (4)	8 (5)	8–16 (7)
actobacillus (homoferm.)	0 (13)	2–32 (12)‖	0 (7)
leomorphic rod (unidentified)		0 (16)	0 (18)
roteolytic rod (unidentified)		0 (5)	0 (5)

* The monocontaminated rats were germfree up to the time of weaning. They were then oculated orally with a pure culture of the indicated organism and maintained as monoconminated stock.

† Antigens homologous for the monocontaminated group.

‡ Number of animals observed appear in parentheses.

§ Range of titers expressed as reciprocal of dilution.

‖ First-generation rats only.

enough protection to maintain the antigenicity of dead indigenous micrococci. This possibility is currently being investigated.

Antibacterial Agglutinin Titers in Normal Sera of Monocontaminated Rats

The agglutinin response of "germfree" rats to monocontamination with pure cultures of viable bacteria is presented in TABLE 3. These monocontaminated animals were in contact with their monoflora through the same natural channels by which a conventional animal is exposed to its body flora. No parenteral treatment was given. Data for germfree and conventional rat sera are included for comparison. The antigens used in each test were homologous for the monocontaminated group.

Conventional rats produced fairly good titers for *Streptococcus fecalis*. *S. fecalis* counts in the cecum of conventional animals usually number log 6 to log 7/gm. dry cecal contents. In comparison, rats monocontaminated with *S. fecalis* have cecal counts of log 9 to log 10. Thus, monocontaminated rats may harbor 100 to 10,000 times more *S. fecalis*/gm. dry cecal contents. The members of the monocontaminated group also tend to produce higher agglutinin titers for this organism than their conventional counterparts. The germfree rats tested negative.

The results with lactobacilli were also interesting. Heterofermentative lactobacilli are the predominating organisms in the intestinal tract of the conventional rat, numbering approximately log 10/gm. dry cecal contents. "Germfree" rats monocontaminated with heterofermentative lactobacilli also harbor a comparable number of these forms. The similarity in *Lactobacillus* population is also reflected in the similar titers observed in the two groups. The titers observed in conventional rats are generally below those seen for streptococci despite the fact that lactobacilli are more numerous. However, lactobacilli are known to be fairly weak immunogenically, which may explain the lower titers observed. Germfree rats had negative agglutinin titers for this organism.

The monocontaminated rats showed positive titers to homofermentative lactobacilli, but the germfree and conventional rats did not. In the previous discussion of TABLE 2 it was pointed out that homofermentative lactobacilli are not generally found in conventional Lobund rats and, of course, these are also absent in germfree animals. It is therefore not surprising to find no agglutinins for these forms in germfree and conventional rats (under certain conditions rats monocontaminated with homofermentative lactobacilli also fail to produce agglutinins; this is considered below and in FIGURE 6).

The pleomorphic rod is the same organism described as the "cecal" organism in my paper "Determination of Germfree Status," elsewhere in this monograph. This rod failed to stimulate agglutinin production in either monocontaminated or conventional rats. The same results were evident in rats monocontaminated with an unidentified proteolytic rod. Neither of these latter two organisms grew well in the monocontaminated animals and neither could be found associated with conventional rats. Under these conditions, no agglutinins were formed. The pleomorphic "cecal" organism was also shown to be a poor immunizing antigen for the rat. A series of parenteral injections of this organism into conventional rats failed to stimulate an agglutinin response.

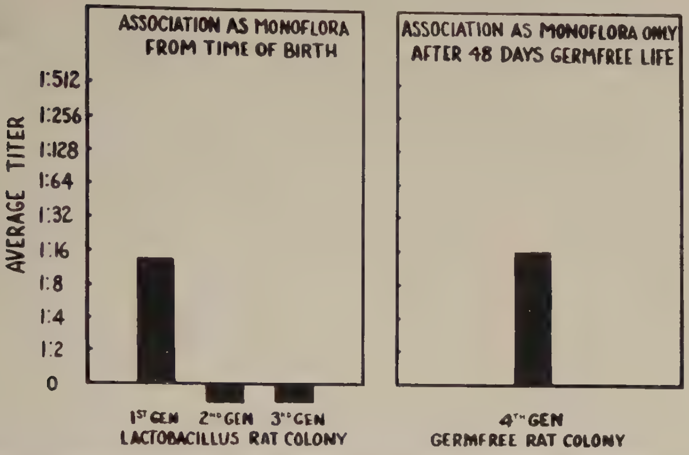

FIGURE 6. Agglutinin titers in rats reared with homofermentative lactobacilli as the sole microbial contaminant.

Agglutinin Titers in Rats Reared Monocontaminated with Homofermentative Lactobacilli Through Several Generations

FIGURE 6 illustrates how the serologic response of germfree animals exposed to monocontamination may be influenced: first, by the organism used; second, by the time at which the germfree host is exposed to contamination; and, third, by the number of generations a germfree animal is removed by cesarotomy from its conventional progenitors. First-generation germfree animals were hand fed on milk formulas, while successive generations were suckled by the germfree mothers.

The first bar in FIGURE 6 represents the average titer of agglutinins produced by first-generation hand-fed germfree rats inoculated orally at birth with a pure culture of homofermentative lactobacilli (the bar is plotted from the corresponding data in TABLE 3). These rats were allowed to breed to form a "*Lactobacillus*-rat" colony extending into the third generation. The second- and third-generation animals were also contaminated with lactobacilli at birth by exposure to and contact with the monocontaminated mother and environment. However, the second- and third-generation rats failed to elicit an antibody response despite the fact that all 3 generations were exposed to the lactobacilli at birth.

In another experiment, a litter of fourth-generation germfree rats was given a delayed oral inoculation of lactobacilli after 48 days of germfree existence. Under these conditions, agglutinins were produced.

The reasons for these differences are not clearly understood. However, one may speculate that they may be related in some way to an embryonic actively acquired tolerance.[9] For example, the second- and third-generation animals in the *Lactobacillus*-rat colony were all born from mothers harboring large numbers of homofermentative lactobacilli during pregnancy. Therefore the contact of the embryos with *Lactobacillus* antigens cannot be dismissed. On the other hand, the fourth-generation germfree rats had no such prior embryonic

TABLE 4

PROPERDIN, COMPLEMENT, AND COMPLEMENT-COMPONENT TITERS IN NORMAL SERA OF GERMFREE AND CONVENTIONAL RATS

Serum No.	Properdin (units/ml.)	Complement (units/ml.)	Complement components (units/ml.)			
			C′1	C′2	C′3	C′4
GF-134	48	50	700	90	700	45
GF-135	48	50	1000	90	1000	45
GF-139	48	50	700	90	700	45
C-1001	48	65	1400	90	1400	60
C-1002	56	80	1400	120	1000	60
C-1006	56	80	1400	120	1000	60

experience. Likewise, as discussed earlier, the conventional parents of the first germfree generation apparently had no contact with homofermentative lactobacilli.

The above speculation becomes less attractive when the question is raised as to why conventional animals do not also develop an actively acquired tolerance to many of the bacteria comprising the body flora.

Properdin, Complement, and Complement-Component Levels in Normal Sera of Germfree and Conventional Rats

The properdin, complement, and complement-component titers of germfree animal sera have been of some interest. Theoretically, the amounts observed in germfree animals should represent levels closer to the base line, since these animals undergo less antigenic stimulation in the absence of a viable microbial flora.

The data presented in TABLE 4 were obtained in collaboration with the late Louis Pillemer of Western Reserve University, Cleveland, Ohio, and were mentioned briefly in one of his papers.[10] There was a tendency for germfree rat serum to be on the lower limits of levels seen in conventional rats. However, few observations were made, and it is likely that no real differences in properdin and complement levels existed between the germfree and conventional rats as run. These titrations were made in germfree rats giving positive agglutination titers with *M. epidermidis* (see TABLE 2). Consequently, the data may not represent levels in minimally stimulated animals.

Summary

Germfree and conventional animals examined to date are comparable in terms of complement, properdin, and at least certain of the heterohemagglutinins; however, they differ in antibacterial agglutinins. Germfree chickens remain free of agglutinins against common intestinal bacteria long after conventional chicks have developed positive titers. Older germfree chicks may eventually develop agglutinins against certain bacteria that are traceable to the diet prior to autoclaving. Germfree rats are also generally free from antibacterial agglutinins readily demonstrated in conventional rats. However, anomalous reactions with micrococci have been observed in germfree rats.

Rats maintained with only single strains of viable microorganisms respond differently in terms of antibody production. The response may be influenced by the organism used, by the time at which the organism is introduced into the previously germfree animal, or by the number of generations by which the animal is removed from cesarotomy.

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ORIGIN OF ANTIHUMAN BLOOD GROUP B AGGLUTININS IN GERMFREE CHICKS*

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The present hypotheses concerning the origin of antihuman blood group A and B agglutinins are contradictory. Furuhata[1] and Hirszfeld[2] consider them to be hereditary, while others such as Dupont,[3] Armangué,[4] Wiener,[5] and Kabat[6] believe that they are acquired. Both positions are adequately discussed by the latter two authors.

Blood group active antigens are widely distributed,[7, 8] being found in mammals and in many of the lower animals. They are also present in some plants and in Gram-negative bacteria. This wide distribution is compatible with the hypothesis that blood group antibodies are the result of inapparent or cross immunization.

The problem of whether antibodies are inherited or acquired is of importance for all so-called natural antibodies. Blood group agglutinins against erythrocytes of numerous animals, including man, have been found to occur in chicks at about the sixteenth day of life and have been thought to be inherited.[9]

Most sera from heterologous species contain species-specific antibodies that will agglutinate all types of human cells.[6] A multitude of antigens are on the erythrocyte surface, and many different antibodies can produce the phenomenon of agglutination. "Natural" antihuman blood group specific antibodies have long been known to occur in chicks.[9a] Antihuman blood group B agglutinins have been found regularly by Uyeyama,[9b] by Iseki *et al.*,[10] and by ourselves.[11] Baer *et al.*,[12] who studied antihuman blood groups A and H(O) antibodies, found these to be present "naturally" in all chicks investigated.

When we began our studies on the formation of antihuman blood group B agglutinins Wagner had already reported his observation on occurrence of agglutinins against rat and rabbit erythrocytes in germfree chicks.[13]

The specificities that must be considered are shown in TABLE 1. In addition, it had been shown by von Dungern 58 years ago[14] that the injection of cow's milk into various animals leads to very potent antierythrocyte antibodies even against cow erythrocytes. Cow's milk casein is fed in large quantities to the bacteria-free chicks used in these experiments.

The means we used to clarify this picture are shown in TABLE 2. In the present study, groups of the same hatchings of Leghorn chicks were raised on a semisynthetic diet free of demonstrable blood group activity, in germfree tanks of the type described by Reyniers elsewhere in these pages and in a conventional

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† Established Investigator of the American Heart Association, Inc., New York, N. Y.

TABLE 1
SEROLOGIC SPECIFICITIES

Interspecies	Intraspecies
Species	Isoantigenicity
Organ	Autoantigenicity
Heterogenetic	

TABLE 2
AGGLUTINATION OF HUMAN BLOOD GROUP B ERYTHROCYTES BY CHICK SERA

	Age (days)	No.	Unabsorbed, not decomplemented	Absorbed,* not decomplemented	Absorbed,* decomplemented
Conventional	28	3302	8	1	2
		3319	8	2	2
		3321	128	16	16
		3322	2	<1	<1
	56	3318	16	4	4
		3323	16	8	8
		3326	32	8	8
Germfree	28	3428	1	<1	<1
		3429	4		
		3432	2		
		3441	<1		
	56	3427	2	<1	<1
		3435	2		
		3443	4		

* One absorption with human blood group O erythrocytes.

environment.[15] While conventional chicks generally possess specific antihuman blood group B agglutinins by the age of 4 or 5 weeks, no such agglutinins could be demonstrated in germfree chicks up to about 60 days of age. Unless stated otherwise, all chick sera were decomplemented for 20 min. at 56° C. and then absorbed for 1 hour with an equal volume of packed, washed human group O Rh^+ erythrocytes.

Blood group B was convenient for us to investigate, first, because we found that almost all our conventional chicks when over 30 days of age had some antibodies against it,[11] and second, because we did not find blood group B activity in the unconcentrated semisynthetic diet.[11, 15] Nevertheless, trace contamination with blood group substances that resist autoclaving is still conceivable because of their ubiquitous nature and their presence in dust, sweat, and droplet excretions of those preparing the diet.*

We have found that *Escherichia coli* O_{86} B:7 possesses extremely high blood group B activity[7, 8, 11] and antigenicity. This bacillus established itself well in germfree chicks, but not in conventional birds.

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* Specific blood group agglutination-inhibiting substances have now been found in some diet concentrates (unpublished experiments).

Figure 1 shows our observations on the formation of antihuman blood-group B specific agglutinins in chicks. Note that the conventional birds form a moderate amount of antibodies, as was to be expected, while germfree chicks form none, at least not up to about 60 days of age (see also table 2). At present we attempt, by studying 3-month-old germfree chicks, to determine whether the trace amount of anti-B titer (about 1:1) in 66-day-old germfree chicks is due to very small amounts of blood group B active material penetrating the germfree barrier. Germfree chicks fed live smooth *E. coli* O_{86} form very potent antihuman blood-group B agglutinins.

Figure 2 shows that the so-called albumin- or incomplete-antibody formation against the blood group B system does not significantly differ from that of saline

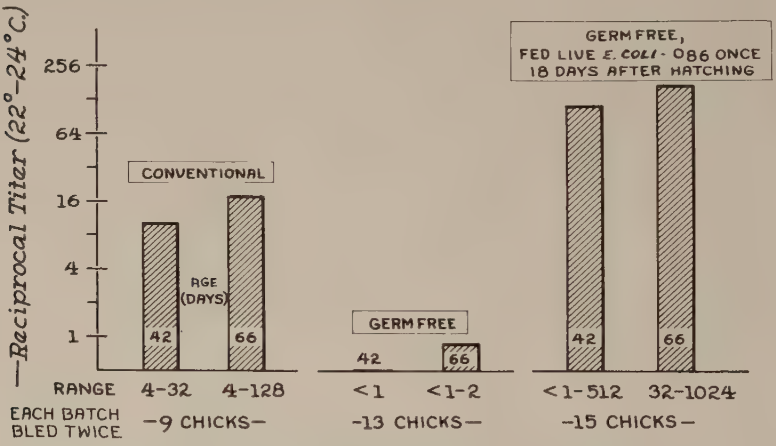
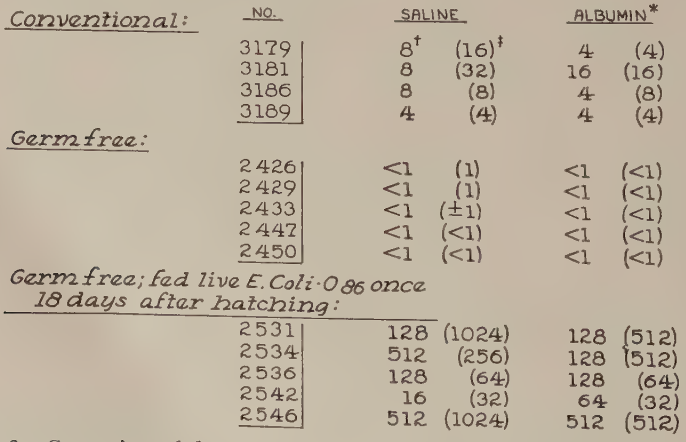


Figure 1. The development of antihuman blood group B agglutinins in Leghorn chicks (one hatch). Each titration was done in triplicate, and the average used.



	NO.	SALINE		ALBUMIN*	
Conventional:					
	3179	8†	(16)‡	4	(4)
	3181	8	(32)	16	(16)
	3186	8	(8)	4	(8)
	3189	4	(4)	4	(4)
Germ free:					
	2426	<1	(1)	<1	(<1)
	2429	<1	(1)	<1	(<1)
	2433	<1	(±1)	<1	(<1)
	2447	<1	(<1)	<1	(<1)
	2450	<1	(<1)	<1	(<1)
Germ free; fed live E. Coli-O86 once 18 days after hatching:					
	2531	128	(1024)	128	(512)
	2534	512	(256)	128	(512)
	2536	128	(64)	128	(64)
	2542	16	(32)	64	(32)
	2546	512	(1024)	512	(512)

Figure 2. Comparison of the saline and albumin agglutination in Leghorn chicks (reciprocal titer at 37° C.). * Twenty-two per cent bovine albumin. † At 42 days of age. ‡ At 66 days of age.

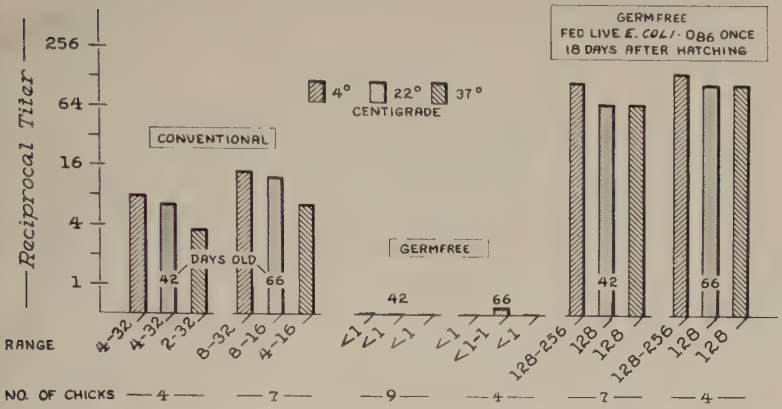


FIGURE 3. Thermal amplitude of antihuman blood group B agglutinins in Leghorn chicks (average titer).

agglutinins. This is important, because presence of incomplete antibodies has been considered by numerous workers to be a characteristic of immune antibody.[6] The very weak saline agglutinins in a few of the 66-day-old germfree chicks are no longer demonstrable in an albumin medium considered to be optimal for the detection of incomplete antibodies.[16]

Finally, FIGURE 3 shows that the thermal amplitude of so-called natural antibodies is essentially the same as that of the immune agglutinins induced by *E. coli* O_{86}. A different thermal amplitude for natural and immune antibodies has been claimed by many.[6] There was no significant difference in total serum proteins and in total weight in the different groups of chicks.

We conclude from these experiments that antihuman blood group B agglutinins in chicks are not inherited, but are aquired early in life.

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DISEASE-FREE LABORATORY ANIMALS AS RELATED TO GERMFREE LIFE

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A discussion of disease-free animals as related to germfree life presents some problems of terminology, as well as the misconception that the rearing of germfree animals is a new, difficult, and expensive procedure. We shall attempt to clarify these particular points.

Let us first define some of the terms commonly applied to the animals that we shall refer to as disease-free in this section of this monograph. The more commonly used terms are disease-free, pathogen-free, specific pathogen-free (SPF), cesarean derived, and "clean" animals. These terms are used interchangeably at present to designate the same animal, and each is somewhat ambiguous or incorrect.

Disease-free, in effect, means "free from symptoms of disease." However, the animals of which we are speaking must be free, not only of symptoms of disease, but also of latent infections and of the carrier status as well.

The term pathogen-free infers freedom from all pathogenic organisms. However, this term becomes incorrect if we accept the tenet that every organism can be pathogenic under certain circumstances. In effect, then, the terms pathogen-free and germfree would be synonymous. This is incorrect, inasmuch as the animals to which we refer have a restricted flora.

The term specific pathogen-free is perhaps more correct, as it implies that the animals so designated are free of specific pathogenic organisms that could be listed. However, since every such colony in the United States could be found to be free of at least one pathogenic organism or disease (infectious ectromelia, for example), every colony could be designated as an SPF colony. This is particularly true because of limited diagnostic facilities, the limitations of diagnostic procedures, and the impossibility of testing every animal in a colony. Therefore, the term specific pathogen-free becomes meaningless when, by common usage, every colony in the United States can be so designated.

Cesarean-derived is a term indicating that the original stock was obtained by cesarean section. This is a good term in that it has been well established that the cesarean technique under aseptic conditions is the only method that can assure that the animals so derived will be completely free from all pathogenic organisms. However, the term does not indicate that the proper environmental controls have been maintained, nor does it indicate the disease status of the progeny of the cesarean-born animals.

Only through years of common usage can the term clean animals come to mean animals other than those not needing a bath. We have not had those years of common usage as yet, so this term, to a great extent, is meaningless. We are therefore faced with the problem of designating a specific type of animal without having a truly descriptive terminology.

In describing the animal in question, whether it be called disease-free, pathogen-free, specific pathogen-free, cesarean derived, or clean, one must recognize that this animal is actually, by usage, a very delicate research tool subject to many physiological and pathological changes caused by genetic and environmental variations.[1] To produce such a tool, properly calibrated or standardized, one must control these genetic and environmental variations by starting breeding colonies with stock of known genetic purity, free from disease-causing organisms. This colony then must be maintained in the proper physical environment on a proper diet, free from exposure to disease-causing organisms. This is not only a description of how these animals are produced, but also a definition of the terms used for designating the animals themselves. Whether they be called "disease-free," "pathogen-free," "SPF," "cesarean derived," or "clean" animals, the term used, to be meaningful, must make clear the fact that the colonies are free from such disease-causing organisms. In addition, any term used to describe these special animals must assure a standardized environment in the form of controlled temperature, humidity, nervous stress, and nutrition, as well as a known and recorded genetic background.

At present few animals in the world could meet such rigid standards, and this, perhaps, explains the confusion in terminology. It is believed, however, that within a few years the commonly used term "laboratory animal," will, by common usage, indicate such a research tool that complies with rigid standards and with variables controlled to that level required for definitive quantitative investigations. This belief is based upon present urgent needs for an improved laboratory animal and also on the enthusiastic reception given by investigators to the disease-free animals produced to date.

One might compare the status of laboratory animals today to that of culture media fifty years ago before standardized dehydrated media became available. The variations in susceptibility and response of individual animals from the various conventional animal colonies today are comparable to the variation in composition of small batches of culture media produced fifty years ago. The development of disease-free animal colonies is a step toward eliminating such variations.

At the present time laboratory animal colonies may be classified into three distinct groups: (1) The germfree colonies, which are the "pure-culture" or "pure-reagent" class of the animal groups; (2) the disease-free colonies that could be classed as the U.S.P. grade of reagents (not quite "pure," but with limited and specified "impurities" present); and (3) the conventional colonies that would then be comparable to the commercial grades of reagents. The lack of standardization of this commercial grade limits its use for highly specialized, definitive procedures. This is also true of animals limited in their use as research tools by the physiological and pathological changes caused by "impurities" in the form of genetic and environmental variations.[2]

This is understandable when one considers that the results of biological procedures using animals are based upon the physiological and pathological changes observed in the animals and caused by the planned stress of the procedure. Similar changes may be caused by variations in the genetic background or environment of the animal. Such changes caused by genetics or environment must be standardized or eliminated in order that they may not be confused with, or even nullify, research results. Planned control of variables is essential in any research tool, and controlled production procedures are necessary for producing such standardized tools. Laboratory animals can be produced with a marked degree of uniformity for use as research tools by similarly well-controlled production methods.

A brief consideration of what these production procedures must entail discloses an interesting fact. While the mass production of disease-free animals is relatively new, the methods used are simply an application of those facts of genetics, epidemiology, nutrition, and general ecology that have been well known and accepted for a great number of years. Let us consider the steps necessary to establish a colony of such animals.

First, the selection of breeding stock of known genetic purity has been simplified by the many years of work accomplished in the area of selective breeding. There is still much work to be done in this area, but the groundwork is well laid, and thirty years of excellent work by many geneticists are behind us.[3, 4]

It has also been well established by years of work on germfree animals that the placenta is an effective barrier against organisms.[5] This makes it possible to obtain germfree offspring from diseased parents by aseptic cesarean sections. It should be noted that this is the only effective way, and certainly a very practical way, to accomplish this task. There is no drug or combination of drugs that will eliminate all organisms from an animal and still maintain the animal in condition for breeding purposes.

The hand-nursing of cesarean-born animals is time consuming and difficult in the majority of rodents, but it can be extremely simple in the guinea pig and larger animals.[6, 7] Again, the techniques required have been established for many years. Fortunately, due to the splendid cooperation of the Lobund Institute, Notre Dame University, Notre Dame, Ind., in supplying germfree and monocontaminate animals to individuals interested in starting disease-free colonies, the tedious job of hand-feeding is seldom necessary in the more difficult rodents. Once a small stock of rats or mice has been established by hand-feeding or from established germfree or disease-free colonies, the use of foster mothers becomes possible. These disease-free foster mothers may be used to nurse cesarean-born offspring from other strains and species. The cross-nursing of species occurs frequently in nature and may be induced readily in an animal colony by destroying the foster mother's ability to identify the strange offspring by their odor. This can be accomplished by the use of cedar shavings for bedding material and the application of some strong-smelling agent on the nose of the foster mother after her own offspring are removed and before the cesarean-born are placed with her. This technique has been used for centuries in nursing orphan lambs on foster mothers.

The epidemiological pattern of infectious diseases has been sufficiently established over the past century to ascertain that spontaneous outbreaks do not occur without contact with a disease-causing organism.[8] A barrier between such disease-causing organisms and the disease-free animals must be established and rigidly maintained. A realistic approach to establishing this barrier may be made if one but recognizes that any insect, fowl, man, or other animal, or anything that they contaminate, is a potential source of disease-causing organisms. This must be a positive approach, undiluted by assumptions of safety because of appearances or small samplings that show no pathogenic organisms. This approach dictates that everything passing through the barrier to the colony room must be treated by proved methods of sterilization and transferred to the colony room without contamination. Air for ventilation must be dustfree and properly filtered. Personnel entering the room must be prevented from carrying pathogenic organisms to the animals by unsterile protective clothing. Aseptic procedures established for human surgery may be well adapted to this area of personnel management.

The need for a controlled physical environment, including temperature, humidity, and nervous stress, has been well established by investigators in the field of ecology.[9] Modern equipment makes it possible and of considerable monetary value to establish such controls in animal quarters.

Nutrition is also a field in which years of work have been accomplished. It has been demonstrated that physiological and pathological changes ranging from poor condition in a few animals to the death of an entire colony can result from a lack of proper nutrients. Experience has shown that the purchase price of feeds can be a dangerous criteria for selection of diets. Proper evaluation frequently shows that the cheapest feed is often the most expensive.[10]

These areas of consideration for developing disease-free colonies that we have grouped loosely under genetic background, disease, physical environment, and nutrition must be considered as a whole rather than as separate entities. This becomes apparent when considering the laboratory animal as a standardized research tool. An animal with the purest genetic background is of little value if it is suffering from malnutrition or is exposed to extremes of environmental temperature. Animals with the purest genetic background and housed in the most ideal quarters are worthless if they are dying of disease.

In summary, we may note that in any area of controlled research resulting in reproducible results, properly calibrated or standardized research tools are a necessity. This is also true in biological procedures performed with an animal as the basic research tool. Animals standardized to a marked degree of uniformity can be produced by applying the knowledge of genetics and ecology that has been available for many years. Such a program is not only possible but extremely practical, for the added costs of establishing a controlled environment are more than defrayed by increased production and lowered mortality in the animals, as well as by reproducible results in research experiments. To be successful, this program must be guided in a practical, common-sense manner by an individual who has specific awareness of bacteriology, epidemiology, disease, genetics, aseptic techniques, animal husbandry and, above all, a psychological understanding of personnel relations and management. Equipment and facilities are of the utmost importance; however, the human element can compromise all. In closing, it is encouraging to note that a disease-free laboratory animal is one of the few products whose production cost is in inverse ratio to its quality. Such an animal of the highest quality is he cheapest to produce.

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Part IV. Use of Germfree Vertebrates in Research

THE GERMFREE ANIMAL AS A RESEARCH TOOL

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Until late in the last century the existence of microorganisms in healthy animals was regarded as of no importance or interest. Such organisms were regarded as saprophytes exerting neither beneficial nor injurious effects on the organism of the host. The year 1885 marked a turning point in this attitude. It was then that Louis Pasteur presented his new ideas. In a preface to a publication by Duclaux,[1] he wrote: "Frequently, during our conversations in the laboratory and for many years now, I have spoken to the young scientists who surrounded me of the interest there would be in nourishing a young animal (rabbit, guinea pig, dog, or chicken) from its birth on pure nutritive materials. By this last expression I mean food products that have been artificially and completely freed of the ordinary microbes. Without wishing to make any assumptions, I shall not conceal the fact that I should undertake such a study, if I had the time for it, with the preconceived idea that life under these conditions would become impossible."*

This was a challenging problem, and it has since been attacked by several investigators. The first attempts were only tentative, but they nevertheless showed that insects and cold-blooded animals could exist and even grow well in the absence of bacteria. Pioneers in this region were Nuttall and Thierfelder,[2-4] Küster,[5-10] and Cohendy and Wollman.[11-13] The last group showed that bacteria-free life with good growth was also possible for goats and guinea pigs.

For many years afterward, as reported elsewhere in this monograph, this field of research received much attention from Reyniers and his co-workers, from Miyakawa and his group, and from Gustafsson and myself. These investigators have now succeeded in rearing germfree animals and maintaining some of them germfree for several generations. The rearing technique has also been improved considerably and now provides invaluable material for applied research in different fields.

Since neither man nor animal is normally germfree, their bacterial flora must reasonably play a role, and the conventional division of bacteria into saprophytic and pathogenic forms only roughly reflects the true situation, since the saprophytic form is not without importance in the organism of animals and man. It may therefore be assumed a priori that the physiological processes must be influenced by these bacteria, just as there is reason to assume that saprophytes play a role in certain pathological processes. Against the background of these considerations two main branches of research have developed: first, investigation of the germfree animal per se and, second, its use as a tool in research. The latter, of course, would not be possible without the former. However, this does not imply that the physiology, structure, and biochemistry of germfree animals must be understood completely before they can be used for applied research, since knowledge of those aspects of importance for the approach to a given problem and the proper use of controls are often sufficient.

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* Editor's translation.

It is thus apparent that the germfree animal is a very important research tool capable of opening up approaches to important biological and medical problems inaccesible with other methods. There is therefore reason to hope that in the near future the different teams working with germfree animals in various parts of the world will shed new light on vital and pathological processes. Those fields in which germfree animals have been or may be used overlap such widely different areas of medicine and biology that every research team must include specialists on histology, physiology, biochemistry, pathology, and bacteriology, as well as other experts on the problem in hand. This is obvious from the following short survey of some of the fields where the germfree animal might be a useful research tool.

Problems that present themselves unsought are those bearing on the humoral and cellular defense mechanism of the body. Some of these appear elsewhere in this monograph.

With the ever-increasing frequency of resistant bacterial strains following in the wake of the increasingly wide use of chemotherapy and antibiotics, questions bearing on defense mechanisms are becoming more and more important. As a matter of fact, it appears that we are headed toward the situation we occupied before the advent of antiseptics. This serious problem may perhaps be solved through better knowledge of the humoral and cellular biological defense of the organism. Possibly the germfree animal will contribute to our knowledge of factors capable of increasing and stimulating the defense mechanism of the body. In nature there seems to be a balance between bacteria and viruses. By stamping out bacterial strains with the aid of chemotherapy and antibiotic therapy we can unveil virus strains. For this purpose the germfree animal provides excellent material. This has been more or less confirmed by certain observations already on record.

A possible relationship, if any, between bacteria and tumors can be elucidated by studies on germfree animals. This problem has already been studied successfully in mice that have a high frequency of spontaneous tumors and in experimental investigations with induced tumors. This is an extremely promising approach to the etiology of these growths. Such investigations alone more than compensate the work and money expended on the production of germfree animals.

Amoebiasis is a sickness that is far more widespread than is commonly supposed. Very interesting observations have been made by Phillips *et al.*[14] on the infection of germfree guinea-pigs with *Endamoeba histolytica*. Experiments have shown that this protozoon requires some unknown growth factor or other factor that is evidently formed by bacteria in the intestinal canal. This gives us an extremely important research tool. The relationship between dental caries and bacterial flora represents another question, important from both a social and individual point of view, that can be elucidated with the use of germfree animals. It has been shown that caries does not develop in germfree animals maintained on a diet capable of producing caries in controls; also that caries occurs on accidental or voluntary infection with a few types of bacteria studied. Further investigation along these lines may enable us to understand the problem of such decay much better than before, and possibly may lead to a much more effective prophylactic and therapeutic treatment of caries.

Biochemistry and nutrition are other fields in which the germfree animal may provide a key to open doors hitherto closed to research. In association with Du Vigneaud, Ressler *et al.*[15] showed, by means of isotope studies on germfree animals, that labile methyl groups are synthesized in these animals to the same extent as in controls. Several investigations of the vitamin requirements of germfree animals have already been made. Gustafsson *et al.*[16] have studied the turnover and nature of fecal bile acids in germfree and infected rats. They showed that after administration of isotope-labeled bile acids the latter were found unchanged in the feces from germfree animals, but were excreted in degraded form by control animals. Monoinfection of rats with *Clostridium perfringens* type E, which *in vitro* breaks down taurin-conjugated bile acids, was found to lead to the fecal excretion of the same degradation products. The turnover rate of bile acids was found to be markedly decreased in germfree animals and partly so in monoinfected animals, and it did not return to normal until some time after the animals had lived in bacterial environments with complete recovery of the normal flora.

The possibility of an infectious component in hemorrhagic shock and in radiation reactions are problems of current interest that can be studied with advantage in germfree animals. Judging from investigations hitherto made, germfree animals appear to be more resistant than conventional ones; however, investigations on these points are still in progress.

Interesting observations by Gustafsson (personal communication) on the increased fat content of the blood in germfree animals has revived the question of relationship between different fat substances and arteriosclerosis. This has opened up a new possibility that may contribute to the understanding of vascular diseases.

Some of the problems that I have mentioned have been studied partly by accidental or voluntary monoinfections. A very comprehensive field of research is offered by monovalent infection, which can be elucidated only by investigation of otherwise germfree animals. It is only in this way that we can study the influence of isolated types of bacteria on physiological and pathological processes. Such investigations may contribute considerably to our knowledge of the sequence of events in many types of mechanisms.

I have dwelt only on some of the fields of research in which the germfree animal is a valuable research tool, but even these few embrace many important medical and biological problems. They all have one feature in common; namely, they can only be solved by investigation on germfree animals. This shows clearly that the germfree animal is a powerful tool in modern biological and medical research. Now that we have, practically speaking, overcome the technical difficulties, we can start research on virgin material. The situation is now that germfree animals as "instruments" can be handed over from centers in different parts of the world to other research workers who are not interested in the technical problems. By conferences including research workers in the field, research can be co-ordinated and arranged along convergent lines pointing to a common goal: the benefit and welfare of mankind.

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A REVIEW OF DENTAL RESEARCH USING GERMFREE ANIMALS

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The oral cavity of man, as well as that of any subhuman animal, is a unique anatomical structure. It provides a salubrious environment for the survival, growth, and propagation of microorganisms. The mouth represents the main entrance to the gastrointestinal tract and, especially when teeth are present, provides a sheltered area—warm, moist, and laden with substrate—that helps render this enclosure a natural incubator for the propagation of microbial life. Many metabolic by-products result, some of which are deleterious to tissues of the mouth.

The marked complexity of the natural microbiota precludes definitive studies of oral disease where microorganisms are intrinsically involved. Considerable effort has been expended to diminish or even to deplete the oral microbial popu-ation. Wide-spectrum antibiotics have been effective in reducing the numbers and kinds of bacteria indigenous to the mouth. Presumably, this has led to a reduction of the amount of tooth decay in some instances, and in lessening he severity of certain soft-tissue lesions around the teeth and elsewhere in he oral cavity. Such efforts and observations are only suggestive, inasmuch as microorganisms can never be completely eliminated in this manner. With an optimal bacterial generation time of only about 20 min., one can expect a repopulation of the stomatological area in a very brief period of time. The only recourse is to attempt to study oral disease in animals reared completely free of all viable microbial forms. In the oral region there are at least three issues that develop lesions caused or influenced by microorganisms: the teeth and their supporting structures; the periodontium; and the mucous membrane of the mouth. All of these have shown presumptive evidence of deleterious nfluence by indigenous or nonindigenous microbic forms.

Enamel and Dentin of Teeth

Tooth decay per se, as well as elementary observations of it, date back to ntiquity. Aristotle, twenty-three centuries ago, observed that those Greeks who subsisted largely on figs had more broken, disintegrated teeth than those who did not partake of this fruit. Countless numbers of additional observa-ions over the centuries, although fragmentary and superficial, have revealed hat dental decay is a very complex disease. E. Magitot and W. D. Miller ointed out the close association of certain "microphytes" and acidogenic acteria, with decay commencing in the enamel but readily progressing to the entin.

It can be stated quite emphatically that dental caries is, and indeed must be, convergent biological phenomenon. As such, it has been possible to conjec-ure that several factors must act simultaneously in order for tooth decay to ccur. These can be defined as constituting at least three separate entities. The stomatic cavity, through which food enters, as the significant beginning of the digestive tract, almost always retains an appreciable amount of food detritus. This residual foodstuff provides an excellent substrate upon which a relatively complex microbiota can exist. Not only do the flora and fauna of the mouth survive but, in this protected natural incubator and well-stocked larder, it is possible for microbial life to propagate in a most luxuriant manner. If the substrate is designated as one definitive factor and the microbial life which it supports as another, then a third and somewhat nebulous factor must be labeled as general resistance on the part of the host or a more specific kind of resistance of the tooth itself to decay.

Of these three factors, that concerned with microbial life and its action as well as reaction in the mouth, although originally the most difficult to control, has now become the most completely controllable. This obviously has been made possible only through the use of the germfree technique and apparatus of the type described earlier in this comprehensive symposium.

For nearly two decades the Zoller Memorial Dental Clinic has collaborated with the Lobund Institute of the University of Notre Dame, Notre Dame, Ind., in a project supported in part by the Office of Naval Research, Department of the Navy, Washington, D. C., designed to study the basic mechanism of tooth decay in a gnotobiotic environment (that is, in an environment in which, within definable limits, known forms of living beings exist). Almost needless to state, the work in this primordial field has been extremely slow and difficult, yet in the last ten years a moderate amount of significant progress has been achieved. As a base line study, the first phase of the project[1] was concerned with rearing white rats completely germfree for 150 days on a diet high in sugar; such a diet ordinarily produces an abundant amount of carious lesions in the molar teeth of conventional control rats. In this negative phase it was possible to demonstrate that the germfree rats developed no caries, not even on a microscopic level. In the second phase of the project, rats on the same diet but otherwise germfree were inoculated with various kinds of bacteria that had shown cariogenic potential in preliminary *in vitro* tests.

To date, only the *Enterococcus* (a strain originally isolated from a carious rat molar) in pure culture was able to produce lesions consistently and somewhat typical of those in conventional control rats.[2]

A third phase is concerned with changes in the dietary substrate, while a fourth phase of the project will be devoted to the addition of various inhibiting agents to a stabilized ecological environment consisting of two constants: a caries-producing microorganism, or a combination of microorganisms and a caries-producing diet as previously demonstrated.[3]

The Periodontium

Even if human teeth could be preserved from carious degradation, they might still be lost or rendered functionless by diseases of the supporting structures. The periodontium, which includes all supporting tissues around a tooth (free and attached gingiva, periodontal ligaments, cementum, and alveolar bone), can and often does become afflicted with generalized or localized destructive processes, especially later in life. The omnipresence of microorganisms in an obviously "mixed culture" around the dentition permits their participation in the pathogenesis of the periodontium in man and certain conventional animals. In this disease, unlike that of dental caries, microbial activity probably and usually plays merely a secondary role but, in a few instances, it may be a primary factor. Thus, bacteria and their noxious by-products are suspected of aggravating any lesion, complicating the conditions, and contributing to the systemic effect of the disease.

About three decades ago even a sluggish amoeba was implicated in periodontal disease, but this has been neither proved nor disproved. At present, attention has been diverted from this lowly scavenger to several bacterial forms found in the gingival "cuff" capable of producing enzymes that may attack the ground substance and other intercellular components of the periodontium. Under certain circumstances such bacteria can elaborate collagenase, hyaluronidase, and even chondrosulfatase.

While the gingival and periodontal pocket forms a veritable test tube favoring survival of many microbial genera in man and certain animals living under conventional (bacterially contaminated) conditions, no justifiable conclusions can be drawn about the etiological significance of microbial life and its bearing on periodontal health or disease. However, under germfree conditions it is within the realm of possibility that the problem can be elucidated or at least more fruitfully studied. Experiments on this matter are under way at our institution in collaboration with the University of Notre Dame. These studies are supported in part by the National Institutes of Health, Bethesda, Md.

Other Lesions of the Oral Cavity

There are several other kinds of lesions occurring in the oral area that are, or may be, associated with microbial activity, and hence might be elucidated by use of the germfree technique. Such other lesions are found not necessarily in the vicinity of the teeth, and hence are related to neither the carious process nor to periodontal disease. Little has been done to study other such soft-tissue disturbances in laboratory animals, thus paving the way for study in a gnotobiotic environment. In the mouth of man, however, there are several naturally occurring localized soft-tissue lesions. Some of these, such as the aphthous ulcer developing in the buccal mucous membrane and the herpetic lesion frequently found at the vermilion border of the lip, are certainly not uncommon, yet relatively little is known of their viral etiology and developmental sequence. Ulceromembranous stomatitis (Vincent's infection) is associated with oral spirochetes and the fusiform bacillus. Again, the etiological relationship is nebulous.

Various forms of osteomyelitis and osteoradionecrosis are additional destructive conditions of the alveolar bone that are important, albeit probably secondary bacterial infections.

The study of both soft- and hard-tissue lesions under gnotobiotic conditions, although extremely interesting, would entail the dual problem of first inducing such nondental lesions in subhuman creatures as the common laboratory animals and, second, of simulating the lesions in otherwise germfree animals with only a single known microorganism or, if this is impossible, with a known but simplified microflora. Definitive results might be expected if the proper conditions could be established for the experiments.

Conclusion

In conclusion, it can be stated that the natural microbiota of the mouth is a veritable jungle of microorganisms, so complex that interrelationships and intrarelationships only can be conjectured. With the advent of a gnotobiotic environment, it now becomes possible to study a known simplified oral microflora and the etiological aspects of oral disease in mammalian hosts.

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Discussion of the Paper

Robert Fitzgerald (*National Institute of Dental Research, Public Health Service, Bethesda, Md.*): We have recently begun some experiments along the lines developed by Orland, and I should like to make a comment in confirmation of his findings. Using both a different cariogenic diet and a different strain of streptococci, we also have observed lesions forming in the teeth of monocontaminated rats within the brief period of two months.

In some of our exgermfree animals, none of which has ever developed carious lesions, we have noticed the deposition of calculus, and I wonder whether Orland has observed this in his own germfree or monocontaminated animals.

Orland: This matter of calculus formation is, I believe, most interesting. The principal point, of course, is how calculus should be defined. Recently, several of us at the National Institutes of Health have discussed the manner in which salivary concretion might be formed. However, in germfree animals I have seen nothing typical of calculus as it is found on human teeth. We have seen a scalelike material that can be easily peeled away from the tooth but, strictly speaking, I do not think that we should call this material calculus.

Question: Does Orland think it would be possible to immunize animals against dental caries by the use of a *Streptococcus*?

Orland: We have not attempted this, and I know of no one who is considering doing so. However, it would be most interesting. We do know of work that was done during the years of World War II in which extensive efforts were made to immunize certain members of the Armed Forces against lactobacilli and possible dental caries, but all of the results proved negative. Thus, while I should very much hesitate to consider this as a fruitful area for experimentation, it should not be dismissed. In other words, the consensus is that immunization of this kind would not very likely affect the growth of microorganisms of the mouth, especially in a situation in which there is no competition from others.

QUESTION: Does Orland believe that *Lactobacillus* is the only organism implicated in dental caries?

ORLAND: No, I do not. Some of the work presented in this monograph emphasizes this very point.

QUESTION: Would you not agree that the proteolytic theory is the most probable, and that therefore the lactobacilli may be inhibited when existing together with the enterococci?

ORLAND: It is quite likely that no single microorganism does but one single thing with regard to tooth disintegration. Almost every microorganism has a battery of enzymes that it can employ under different circumstances. The evidence seems to indicate that lactic acid alone is insufficient to account for typical dental caries, and that something else must occur for the penetration and disintegration of the dentin or organic substance.

FITZGERALD: On this last point I should like to point out that the microorganism being used in our dental caries research is not an *Enterococcus* in the strict sense of the word; it is a nongelatinase-producing nonproteolytic alpha type of *Streptococcus*, and our lesions have developed when it has been used as a monocontaminant. Thus it may be that what has been termed proteolysis is unnecessary for the formation of dental caries.

A COMPARISON OF THE DEVELOPMENT OF SOME RAT AND MOUSE HELMINTHS IN GERMFREE AND CONVENTIONAL GUINEA PIGS

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Introduction

One of the most intriguing aspects of a host-parasite relation is that of specificity. It is a well-documented fact that a particular animal species may have a parasitic fauna peculiar to itself, and that such parasites may do only poorly or even fail to develop in another species. Germfree animals are uniquely suited for the study of both the development of a parasite and the defense mechanisms of the host, particularly when an abnormal host-parasite relation is involved. The effect of the presence in a conventional animal of the usual large array of associated microorganisms on such a relation is unknown, and the information acquired from germfree hosts may provide some insight into their role.

For several years this laboratory has been engaged in studying the behavior of parasitic protozoa in germfree guinea pigs (Phillips *et al.*, 1955). With the development of successful techniques for the axenic cultivation of a variety of parasitic nematodes to the infective stage *in vitro* (Weinstein and Jones, 1959), it now has become possible to study these helminths in germfree hosts.

Since germfree animals have been little studied in this connection, there is, of course, a paucity of information concerning the development of nematodes in these animals. However, in a survey of various species of germfree animals at the Lobund Institute, University of Notre Dame, Notre Dame, Ind., Phillips (in press) found adult specimens of the dog ascarid *Toxocara canis* in the jejunum of 2 germfree dogs 118 days after their delivery by cesarean section. Presumably these infections had been acquired *in utero* from larvae migrating in the mother. This finding, together with the demonstration by Weinstein and Jones (1959) that certain intestinal nematodes can be reared to the fifth stage axenically, would indicate that living microorganisms per se are not essential for the growth and development of these parasites. Whether microbial activity is essential for reproduction has not been ascertained. Phillips did not find *T. canis* eggs in the intestine of the germfree dogs. As yet, reproduction of parasitic nematodes in axenic cultures has not been obtained. While there is reason to believe that this will occur when the necessary nutrients and physicochemical conditions are provided *in vitro*, reproduction in germfree hosts could afford direct evidence in this regard.

Axenically reared (germfree) infective larvae of the rat parasite *Nippostrongylus muris* and the mouse parasite *Nematospiroides dubius* were among the helminths available, and it was of interest to determine the fate of these nematodes in the germfree guinea pig. The conventional guinea pig is considered an abnormal host for these rat and mouse parasites; these nematodes rarely reach the adult stage in the intestinal lumen. The development of adult worms in the germfree abnormal host could have interesting implications, not only with regard to the relation of bacteria to parasite development in the gut of the host, but also with regard to the nature of host specificity.

MATERIALS AND METHODS

Maintenance of Germfree Animals

Germfree guinea pigs were obtained by cesarean section of near-term pregnant conventional dams. The latter, for the most part, were discards from stock colonies of the Hartley strain, the NIH strain, and hybrids of the two lines. After delivery in a steam-sterilized Reyniers Germfree Operating Unit (Reyniers *et al.*, 1946) the young were transferred to an attached sterile Reyniers Germfree Rearing Unit.

The techniques for feeding, handling, and maintaining the germfree guinea pigs followed rather closely those described by Phillips *et al.* (1959). The germfree animals were fed twice a day on Phillips' diet L-445, which contains 100 gm. ground Purina Laboratory chow,* 100 gm. rolled oats, 15 gm. dried yeast, 10 gm. dextrose, and 5 gm. sodium chloride (iodized) in 1 liter of boiling water. Before introduction into the unit, this diet was sterilized in the attached autoclave for 20 min. at 250° F., following 5 min. of free-flowing steam. Thiamine hydrochloride and ascorbic acid solutions in ampules were sterilized along with the diet and were added at one of the daily feedings at the rate of 0.5 and 10 mg., respectively, per animal. The excess beyond the reported need of the animal was added to compensate for the likelihood of some destruction of the vitamins during sterilization. Sterile water also was available to the animals. This diet is referred to here as the germfree type of diet. Conventional guinea pigs used as controls were maintained on either this diet or on our usual stock diet of pelleted guinea pig ration and fresh kale, and were kept in standard metal animal cages.

When conventional animals were maintained on the germfree type of diet, the food was sterilized, covered immediately with aluminum foil, and placed in the refrigerator, where it remained except for the short periods required to remove portions for feeding. Usually the animals ate these portions within a few minutes. Thus, until the conventional guinea pigs actually ingested the food, it contained few if any living bacteria.

Checks for the maintenance of sterility followed the techniques used by the Lobund Institute (Reyniers *et al.*, 1946). Several samples each of fresh feces, food, and various wastes were taken at least once a week and incubated for at least 2 weeks in thioglycollate broth and blood-brain-heart agar at room temperature, 37° and 55° C. Samples were also tested in Sabouraud's medium for the presence of fungi. Bacteriological samples were always taken when the last germfree animal was removed from a unit and an experiment was terminated. Tests were not performed to ascertain the presence of viruses.

Air entering the germfree units was filtered twice through a medium of spun glass. Supplies, instruments, and other such materials were sterilized by steam in the attached autoclave prior to being brought into the unit. Inocula, which, of course, could not be steam-sterilized, were brought into the units in sealed glass ampules through a trap of 2 per cent mercuric chloride solution. These inocula were bacteriologically sterile prior to their introduction into previously sterilized ampules, the outer surfaces of which were then sterilized.

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* Produced by Purina Mills, Buffalo, N. Y.

Preparation and Administration of Larval Inocula

The filariform larvae of *N. dubius* and *N. muris* used in most of the experiments were reared under axenic conditions, using a mass-culture technique (Jones and Weinstein, 1957; Weinstein and Jones, 1959). Eggs of each species were obtained from the feces of heavily infected stock animals; *N. dubius* from mice and *N. muris* from rats. The mice were naturally infected with the dwarf tapeworm *Hymenolepis nana* and the pinworms *Syphacia obvelata* and *Aspicularis tetraptera.* Small numbers of the eggs of these species accompanied those of *N. dubius* eggs in the isolation procedures.

Eggs were washed by sedimentation, concentrated by centrifugal flotation in saturated salt solution, washed in sterile saline, and then axenized in a solution of 1.25 per cent sodium hypochlorite in 0.8 per cent saline. The eggs then were washed in several changes of sterile saline, tested for sterility, and inoculated into a culture medium on the day they were isolated. In all cases the medium consisted of 50 per cent chick embryo homogenate (Weinstein and Jones, 1959), which contained, per ml., 2.6 mg. liver concentrate, 300 units of penicillin, and 300 μg. of streptomycin.

Cultures were prepared in 750-ml. Erlenmeyer flasks, each containing 20 ml. of medium. They were incubated at 26° C. for 7 to 8 days and then tested for sterility. The filariform larvae that developed were harvested as follows: The contents of each flask were centrifuged at slow speed in conical tubes and the supernatant fluids discarded. The larvae then were washed in sterile saline and pooled, at which time a sterility test was made. A magnetic stirrer was used to obtain a uniform suspension, and the number of filariform larvae per milliliter was determined. Appropriate aliquots then were transferred to small conical tubes and centrifuged slowly to sediment the larvae, which were transferred, in a minimum amount of fluid, to 5-ml. glass ampules, which were flame sealed. A few ampules from among those used in each experiment were chosen at random for sterility tests. The remainder was stored at 10° C. and used the following day to infect animals.

The ampules containing germfree larvae were introduced into the germfree unit through the germicide trap. Both germfree and conventional guinea pigs were inoculated in the same manner and from a single pool of larvae in each experiment. The larvae of *N. muris* were injected subcutaneously, and those of *N. dubius* were administered orally by means of a long blunt-tipped needle attached to a syringe. Conventional control animals (the normal hosts) were infected in a similar manner.

In one experimental series in which only conventional guinea pigs and *N. dubius* larvae were used, the larvae were nonaxenic. They were prepared for experimental use from feces-charcoal cultures prepared in the usual manner. Filariform larvae were isolated by Baermann apparatus 5 to 7 days following preparation of the cultures. The collected larvae were washed thoroughly and, after dilution counts were made, aliquots were taken for the inoculation of conventional guinea pigs and mice.

Necropsy and Examination of Experimental Animals

In some instances, fecal samples were examined for eggs and worms in order to obtain evidence of the first appearance of an infection. Microscopic examination was made of saline mounts and of concentrates prepared by centrifugal flotation in saturated salt solution.

In order to determine the ability of the eggs to develop to the filariform stage, charcoal cultures were made from the feces of germfree guinea pigs that passed *N. dubius* eggs.

All animals were necropsied and examined, using the following procedures: The animals were etherized, and samples of various organs were removed for formalin fixation. The entire digestive tract was then isolated, the stomach, small intestine, cecum, and large intestine being separated in dishes containing saline, and refrigerated. The small intestine of each animal was examined in its entirety except for a few small pieces removed for fixation. Samples were placed on a large slide, slit open, and the surface examined for worms and nodules under a dissecting microscope. In many instances the worms were removed to a dish for later detailed examination. Finally, each piece of intestine was compressed between 2 slides and examined to detect small larvae embedded in the tissue.

The stomach, cecum, colon, and rectum were also slit open, and the contents diluted in saline and examined for worms. Only portions of the walls of these organs were observed microscopically. In the case of *N. muris*, fresh bits of lung tissue were compressed between slides and examined for larvae.

RESULTS

Nippostrongylus muris

This parasite normally invades the host through the skin and is carried to the lungs, where it undergoes further development. It then migrates up the trachea and down the esophagus to the small intestine, where it completes development.

Six germfree and 6 conventional guinea pigs were inoculated subcutaneously with *N. muris* in dosages varying from 2000 to 20,000 infective larvae per animal. In order to obtain an early evaluation of the infection, 1 conventional and 1 germfree animal were necropsied on the third day after inoculation; the remainder of the animals were examined on the tenth day. Two conventional rats (controls) were inoculated with 4000 larvae each and necropsied on the eleventh day postinoculation.

The findings are presented in TABLE 1. As is apparent from this table, no worms were recovered from the intestines of conventional guinea pigs. In the case of 2 of the 6 germfree guinea pigs, however, worms were found in the

TABLE 1

DEVELOPMENT OF *NIPPOSTRONGYLUS MURIS* IN YOUNG GERMFREE AND CONVENTIONAL GUINEA PIGS

Estimated number of filariform larvae inoculated per animal	Number of worms recovered from small intestine		
	Guinea pigs*		Rat controls†
	Conventional	Germfree	
2,000	0	0	—
4,000	0	0	(*a*) 3,361; (*b*) 1,325 (adults)
8,000	0	28 (adults, eggs present)	—
10,000	0	0	—
15,000	0	33 (fourth-stage worms)	—
20,000	0	0	

* The guinea pigs given 15,000 larvae were necropsied on day 3, the rest on day 10.

† The two rats were necropsied on day 11. All of the intestine of rat *a* and only a portion of that of rat *b* were examined.

small intestine. Fourth-stage larvae were recovered from the animal that had been necropsied on day 3. This was quite consistent with the normal growth of this parasite for the time elapsed since inoculation. These worms were sufficiently differentiated so that males and females were readily distinguished. In the other positive germfree guinea pig necropsied on day 10, normal, mature male and female worms had developed. The females contained fertile eggs in the uterus, and many eggs, normal in appearance and undergoing cleavage, were found in the guinea pig's feces.

It was of interest that larvae were readily found molting to the fourth stage in press preparations of the lungs of the germfree guinea pig necropsied on day 3. In the conventional counterpart animal, however, none of the third-stage larvae were in molt. Sections of lungs from inoculated germfree and conventional guinea pigs are presented in FIGURES 1 and 2. It is apparent that a cellular response to migrating *N. muris* larvae occurred in the germfree as well as in the conventional animal.

Numerous nodules containing larvae, associated with marked cellular infiltration, were observed in sections of the skin of both germfree and conventional animals. A study of the comparative histopathology is under way to ascertain whether there are differences in tissue response between the two kinds of animals.

Nematospiroides dubius

The host (normally, the mouse) becomes infected with this parasite by swallowing infective third-stage larvae. The larvae burrow into the intestinal mucosa, undergo further molting and development, then break out into the

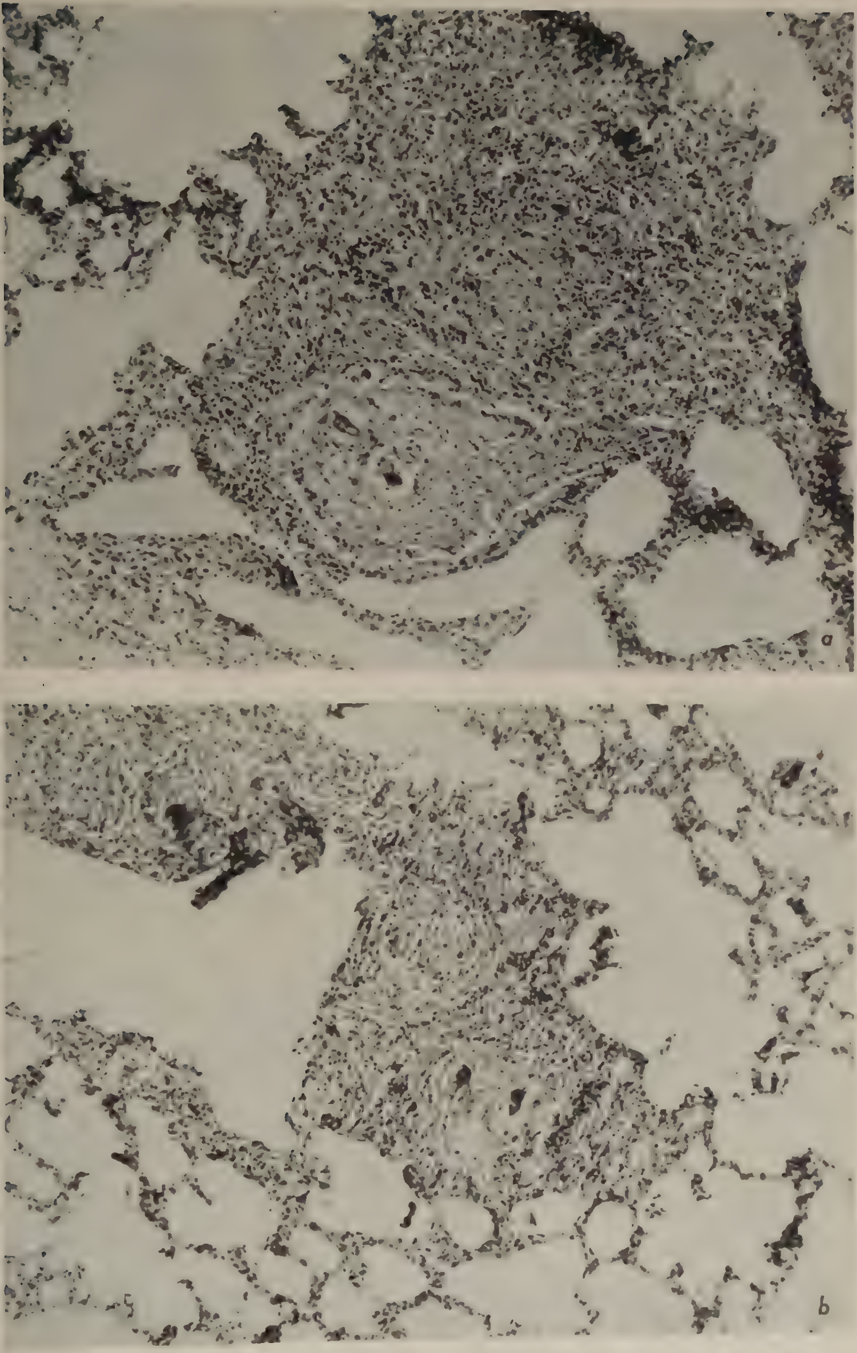

FIGURE 1. Sections of migrating larvae of *Nippostrongylus muris* in lung tissues of guinea pigs: (*a*) from conventional animal, (*b*) from germfree animal. ×105.

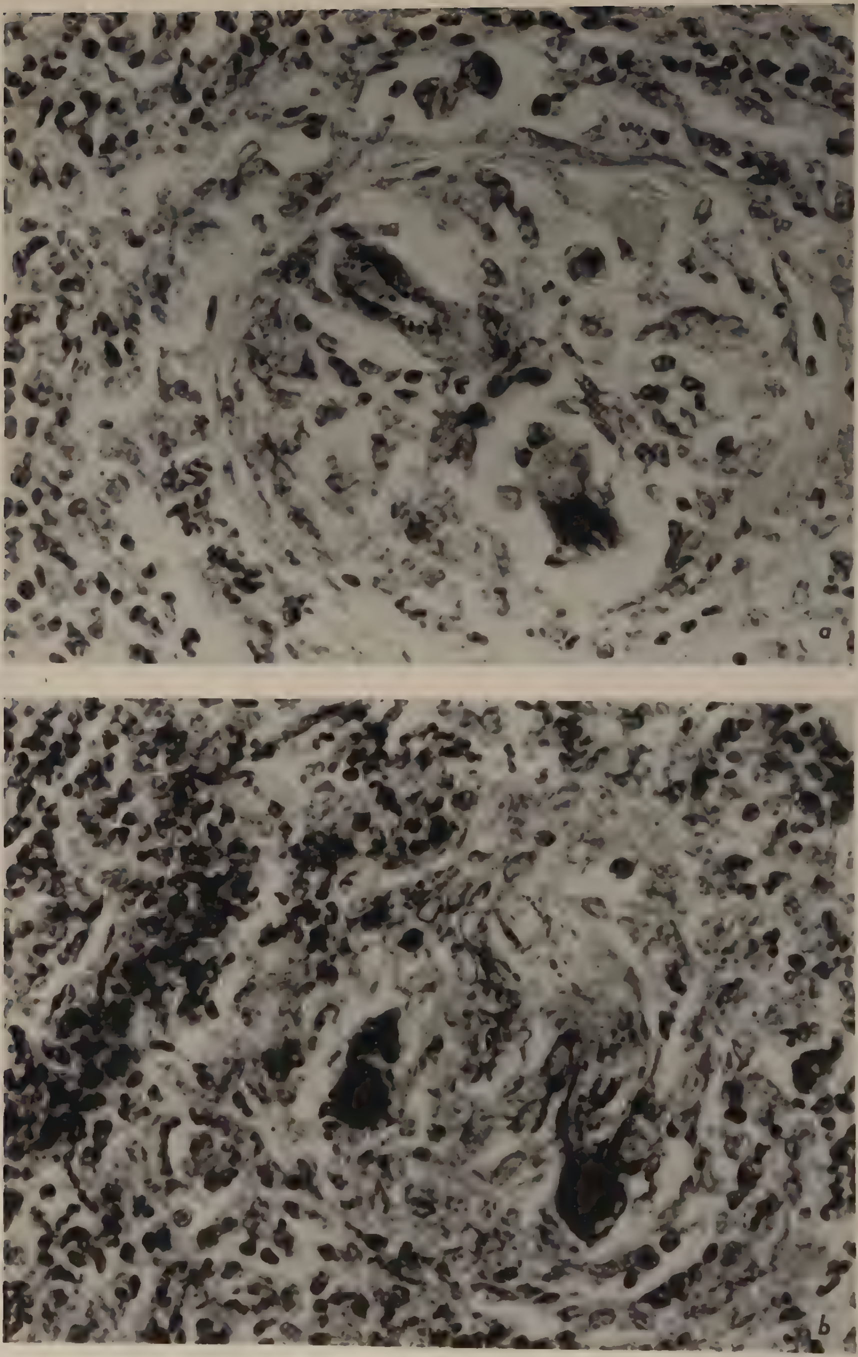

FIGURE 2. Sections of migrating larvae of *Nippostrongylus muris* in lung tissues of guinea pigs: (*a*) from conventional animal, (*b*) from germfree animal. Greater magnification than in FIGURE 1. ×415.

TABLE 2

DEVELOPMENT OF *NEMATOSPIROIDES DUBIUS* IN YOUNG GERMFREE AND CONVENTIONAL GUINEA PIGS NECROPSIED ON DAY 17 POSTINOCULATION

Estimated number of filariform larvae inoculated per animal	Number of worms recovered from small intestine		
	Guinea pigs		Mouse controls
	Conventional	Germfree	
1500	0	67 (4.4 per cent)*	—
3000	0	493 (16.4 per cent)†	—
250	—	—	(*a*) 74 (29 per cent) (*b*) 34‡

* Of these, 41 were fifth-stage worms, including females with eggs; also, there were 3 adult female *Hymenolepis nana* with normal eggs.

† Of these, 476 were fifth-stage worms, including mature females with fertile eggs; also, 14 *H. nana* were recovered.

‡ Only a portion of the intestine of mouse *b* was examined for worms. The worms in both mice were mature, and fertile eggs were present in the uterus of the females.

lumen again, where they become adult worms. Baker (1954) has published a description of the course and pathology of the infection.

Experiment No. 1. The first experiment with this parasite involved only 2 germfree and 2 conventional guinea pigs. However, as is apparent from TABLE 2, the results in the 2 kinds of animals were strikingly different. Whereas no worms were found in the conventional animals given either 1500 or 3000 infective larvae, significant yields of adult parasites were recovered from the germfree guinea pigs. Of the 493 worms found in the small intestine of the one given the heavier dose, 476 were at the fifth stage, many mature females containing fertile eggs. The percentage return was about one half that obtained in the normal host, the mouse, although the yields in that species were lower than usual.

Fecal pellets obtained from both of the germfree guinea pigs on day 10 following infection contained embryonating *N. dubius* eggs. In addition, a mature female worm with many fertile eggs in the uterus was embedded in one of the pellets. Eggs also were found in the feces of these animals when examined on the day 13. Charcoal cultures were prepared with these fecal specimens, and many normal filariform larvae were recovered from the cultures 5 days thereafter. Feces from the conventional pigs were examined by smear and by salt flotation at the same time as in the germfree animals. No eggs were recovered. A control mouse examined on day 10 had eggs in the feces.

FIGURE 3 presents cross sections of the small intestine of one of the germfree animals. In the lumen can be seen a portion of a mature fertile female *N. dubius*. In the higher magnification (*b*), segmenting eggs can be noted in the worm. These worms were distributed throughout the entire length of the small intestine, and appeared to be no different from the ones recovered from the mouse.

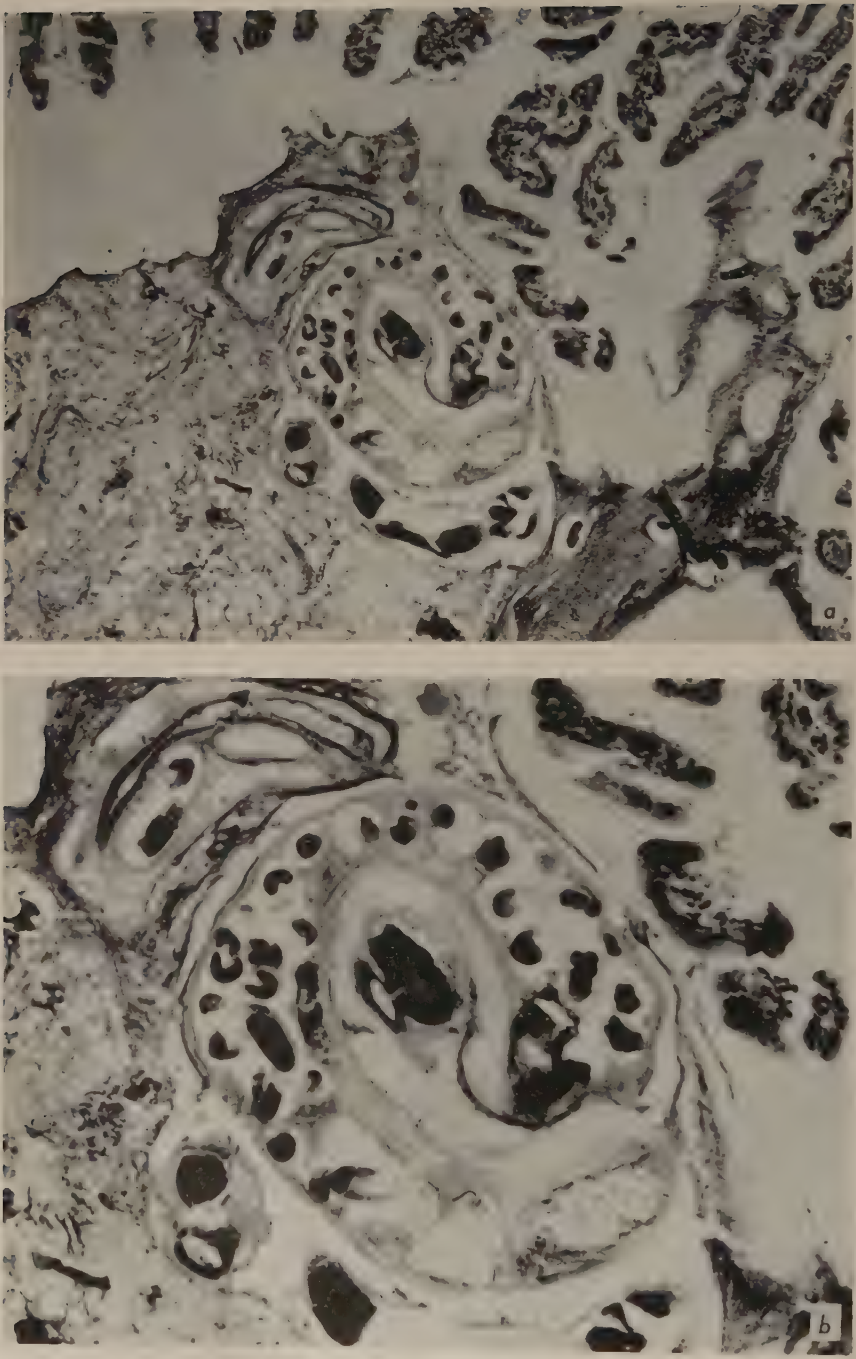

FIGURE 3. Sections of mature female *Nematospiroides dubius*, containing fertilized and segmenting eggs, from the gut of a germfree guinea pig. (*a*) ×75, (*b*) ×150.

As indicated, not all the worms found had developed to adults; some encysted immature and degenerating larvae were recovered. FIGURE 4 shows two magnifications of a worm, probably at the fourth stage, deep within the gut wall of a germfree animal and surrounded by an area of marked cellular infiltration.

Of considerable interest was the fact that several adult *Hymenolepis nana* were recovered from the intestines of the germfree guinea pigs. As pointed out above, a few eggs of this parasite accompanied the *N. dubius* eggs used to inoculate the cultures. FIGURES 5 and 6 show sections of the gut of a germfree guinea pig containing immature and gravid proglottids of *H. nana*. In FIGURE 6*b*, hooklets can be discerned in some of the eggs that were in a gravid proglottid. Morphologically normal *H. nana* eggs also were seen free in fecal smears prepared from the colons of these animals. Neither tapeworms nor eggs were found in the conventional guinea pigs inoculated in this experiment.

Experiment No. 2. Because of the fact that the germfree guinea pigs were cesarean-delivered and maintained on a diet different from that ordinarily given conventional animals, the possibility of an influence of such differences was checked. An experiment including conventional animals maintained on the stock diet, conventional animals maintained on the germfree type of diet, and cesarean-delivered conventional (contaminated) animals maintained on the germfree type of diet was devised. Each of the 11 animals involved was given an estimated 3000 infective *N. dubius* larvae. The latter were reared by the charcoal-culture technique. This, theoretically, would provide larvae that had developed under more "normal" conditions.

As is apparent from TABLE 3, development of the parasite in the various "contaminated" animals was negligible. While the numbers of animals were small, the results were quite uniform, regardless of the test combination. The fact that the germfree animals listed in TABLE 2 had been reared on a special diet or had been cesarean delivered did not appear to be the underlying reason for the successful development of *N. dubius* in these animals. It is to be noted that a few worms did manage to stay alive and underwent some development in the conventional animals. However, only rarely was a fifth-stage worm found. The fact that a total of 33,000 larvae inoculated into 11 guinea pigs resulted in the finding of but a single fertile female worm indicates what a refractory host this animal normally is for *N. dubius*.

Experiment No. 3. A final series of inoculations of *N. dubius* larvae into germfree and conventional guinea pigs was conducted. This was done to extend our observations on the development of this parasite in the germfree abnormal host, as well as to check the possible effect that an age difference might have. While all previous experiments had been conducted with young animals, there was a little variation in age, and it was of interest to determine whether a difference of a few weeks might be important. An average of 1500 axenic filariform larvae from a single lot were administered to each of several germfree and conventional guinea pigs, either 1 week or 1 month of age. Approximately 80 *H. nana* and 10 pinworm eggs, respectively, were also present in the inoculum given each animal. The results of examination of the small intestines of the animals 11 days following infection are shown in TABLE 4.

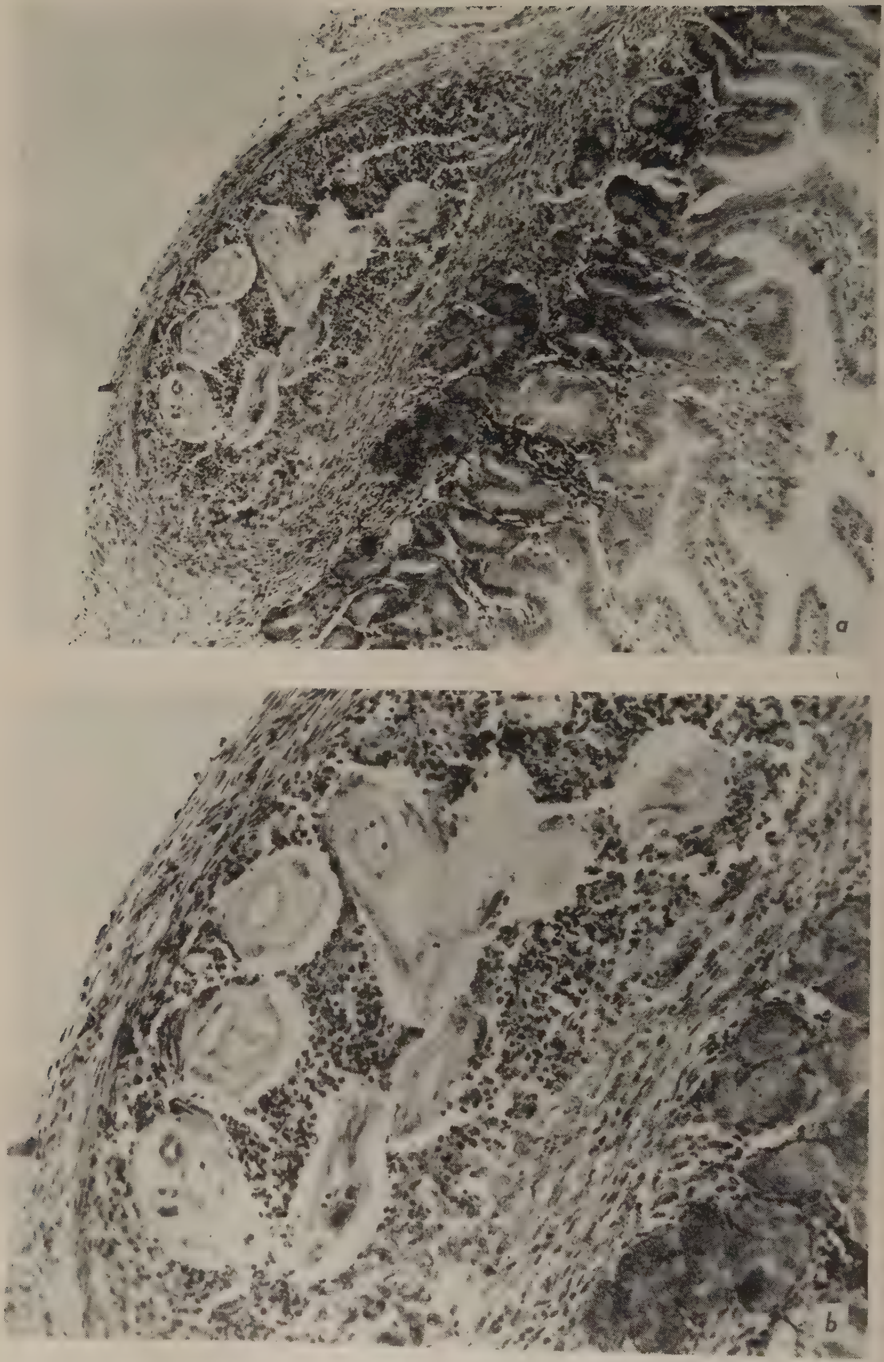

FIGURE 4. Section through *Nematospiroides dubius*, probably a fourth-stage worm, in the wall of the gut of a germfree guinea pig. Note cellular infiltration. (*a*) ×105, (*b*) ×205.

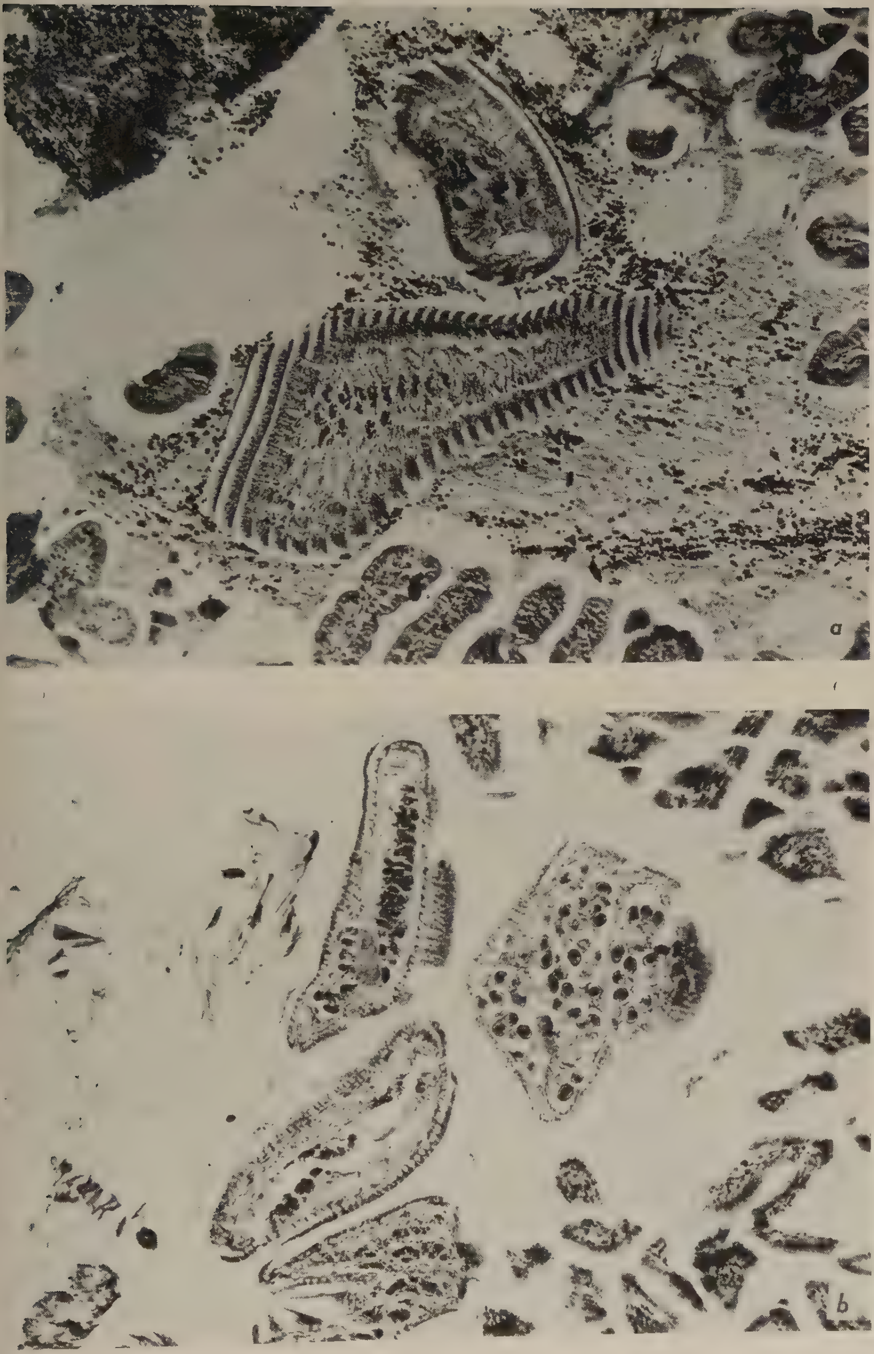

FIGURE 5. *Hymenolepis nana* in sections of gut of germfree guinea pigs (17-day infection): (*a*) section through immature and young proglottids; (*b*) sections through developing and gravid proglottids. ×105.

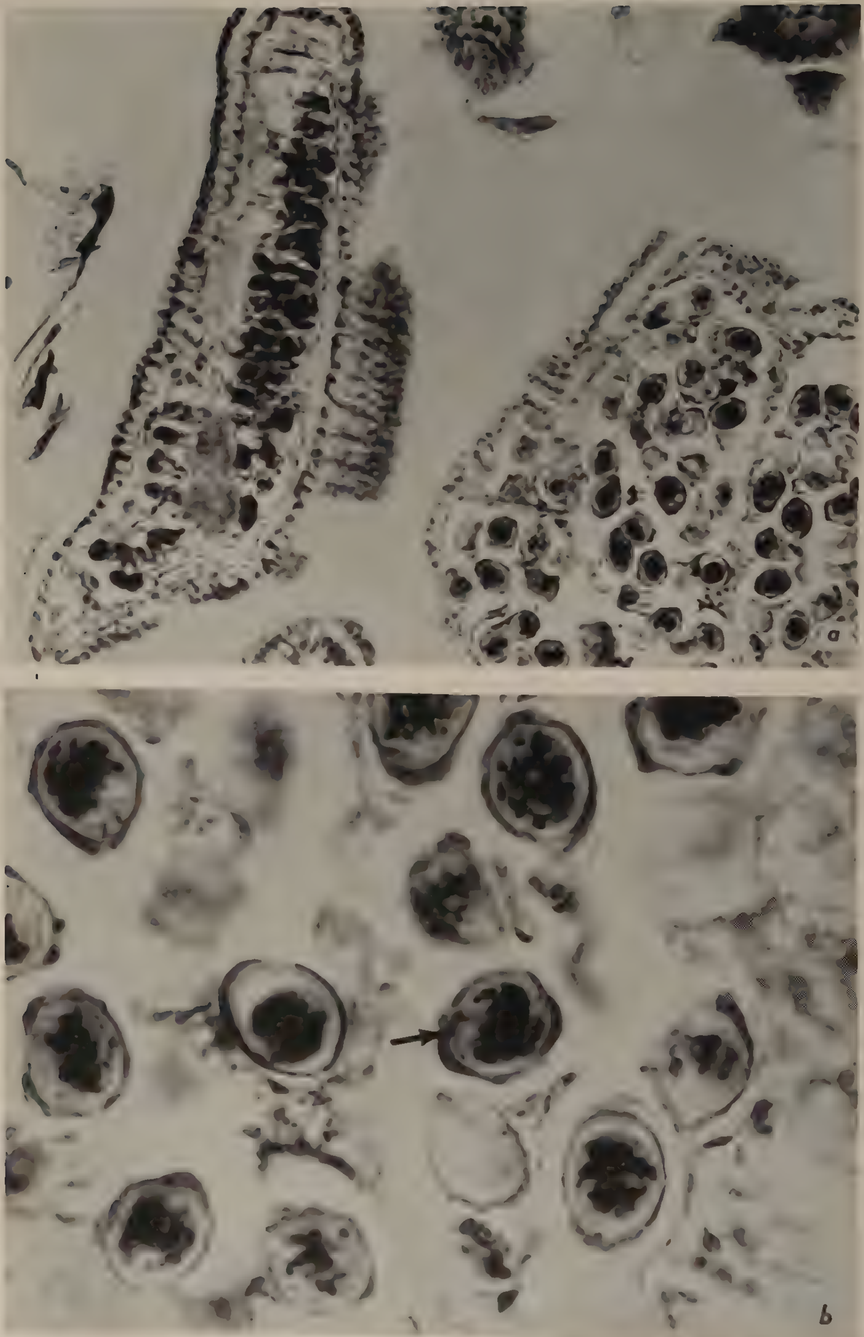

FIGURE 6. *Hymenolepis nana.* (*a*) The same section of the gut of a germfree guinea pig shown in FIGURE 5*b*. ×205. (*b*) Portion of gravid proglottid enlarged to show some of the hooklets in the eggs (arrow). ×775.

TABLE 3

DEVELOPMENT OF *NEMATOSPIROIDES DUBIUS* IN YOUNG CONVENTIONAL GUINEA PIGS REARED ON THE SAME DIET AS GIVEN GERMFREE ANIMALS

Number of animals	Type of delivery	Diet	Estimated number of filariform larvae per animal*	Total number of worms in gut†	Remarks
3	Cesarean	GF‡	3000	1, 4, 0	Fourth, and fifth-stage worms. Only one of the females contained fertile eggs.
4	Conventional	GF	3000	10, 0, 8, 1	
4	Conventional	Stock	3000	0, 17, 0, 0	
3 mouse controls			100	87, 111, 105	Almost all females contained fertile eggs.

* Larvae developed in charcoal cultures (nonaxenic).
† All animals necropsied on day 12.
‡ GF = same sterilized diet fed to germfree guinea pigs.

TABLE 4

DEVELOPMENT OF *NEMATOSPIROIDES DUBIUS* IN YOUNG GERMFREE AND CONVENTIONAL GUINEA PIGS OF DIFFERENT AGES

Type of animal	Number	Age of animals at inoculation	Estimated total larvae received*	Total worms recovered from small intestine†	Remarks
Conventional	8‡	1 week	12,000	74 (0.6 per cent)§	Majority immature and dead; a few live fourth and fifth-stage worms; no fertile eggs
Germfree	7	1 week	10,500	1,267 (12 per cent)§	Majority mature fifth-stage worms; fertile eggs present
Conventional	5	1 month	7,500	9 (0.1 per cent)	All fourth-stage worms; some were dead
Germfree	5	1 month	7,500	724 (9.6 per cent)	Majority mature fifth-stage worms; fertile eggs present
Mouse controls	3	adult	450	383 (85 per cent)	Mature fifth-stage worms; all females contained fertile eggs

* Guinea pigs received 1500 axenic filariform larvae each; mice received 150 larvae each.
† Animals necropsied at 11 days.
‡ Five reared on GF type of diet, 3 of these cesarean-delivered.
§ Some cysticercoids and young *H. nana* tapeworms recovered.

Again in evidence was the marked difference in susceptibility between the germfree and conventional guinea pigs. Among the 1-week-old animals, less than 1 per cent of an estimated total of 12,000 larvae given to 8 conventional (contaminated) animals was recovered. The great majority of these was immature and dead. Five of the 8 animals had been reared on the germfree type of diet, and 3 of them had been cesarean-delivered, again as a control on these possible factors.

In contrast, there was a 12 per cent recovery of worms (range 4.3 to 23.5 per cent) from the 1-week-old germfree guinea pigs, and 9.6 per cent (range, 3.6 to 20.0 per cent) from those 1 month old. In the former group, 71 per cent of the worms recovered were normal mature adults free in the lumen; in the older group 56 per cent were similarly developed and located. The less-differentiated worms were embedded, for the most part, in nodules in the intestinal wall.

There did not appear to be any significant sex difference in the susceptibility of the germfree animals to infection. In the 1-week-old group the average number of worms recovered from 3 males and 4 females was 184 and 178, respectively. In the 1-month-old animals the average recovery from 2 males and 3 females was 116 and 164, respectively. As in experiment 1, the nematodes were distributed rather uniformly and without apparent pattern throughout the entire small intestine.

Developing *H. nana* were recovered from both the conventional and germfree animals. Of the 13 conventional animals examined, 2 contained 2 cysticercoids each, while another had 7 immature tapeworms in the lumen. These 3 animals were all 1 week old. No evidence of tapeworm infection was found in the other 10 conventional animals.

Only 1 immature tapeworm was found among the 5 one-month-old germfree animals. However, a total of 29 immature tapeworms was found in 6 of the 7 one-week-old guinea pigs (range, 1 to 8 per animal). No cysticercoids were evident. Fourteen of the worms were measured, and they ranged from 2 to 13 mm. in length. The failure to find mature proglottids and eggs in the germfree animals, as was the case in experiment 1, is undoubtedly related to the fact that the postinoculation period in this experiment was only 11 days, as compared to 17 days for the earlier one.

No evidence of pinworm infection was seen in any of the animals.

Discussion

The finding of an increased susceptibility in the germfree guinea pig to "abnormal" helminth infections, as compared to the conventional animal, does not, of course, immediately increase our understanding of the factors that underlie host specificity. It does, however, emphasize the potential value of the germfree animal in the study of the effects that microbial associates may have upon a given infecting organism, both directly and indirectly through their possible "conditioning" of the host.

The great majority of the larvae of *N. muris*, a rat nematode, apparently experienced the same fate in the germfree as in the conventional guinea pig as reported for the latter animal by Lindquist (1950); that is, they were trapped and destroyed in the skin and the lungs. However, the facts that molting larvae were observed in the lungs and that a few fertile adults were obtained indicate, of course, that some of the parasites were successful. Haley (1958) found that a rat strain of *N. muris* could eventually be adapted to the hamster, ordinarily a very poor host, by successive hamster passages of larvae derived from the few worms that developed in the latter. This suggests that the worms that succeeded in the germfree guinea pig might have come from larvae genetically different from, or more resistant than, the rest, and that it might be possible similarly to develop a guinea pig strain by repeated passage through germfree guinea pigs. The comparative histopathology of infection with such a strain in the germfree and conventional abnormal and normal hosts could shed much light on the phenomena associated with natural resistance to tissue-invading parasites.

The question arises, of course, as to why a few (or at least demonstrable numbers) of the larvae did not achieve maturity in the conventional guinea pigs as well. Are the tissues of the latter more resistant from an active immunological standpoint, or are they physiologically less suitable than those of the animal that does not carry (or that has not reacted to) microbial infection?

The relatively high yields of mature worms in the germfree guinea pigs inoculated with the mouse parasite *N. dubius* is of considerable interest. The fact that this parasite has a more direct migratory cycle and does not pass through such tissues of potentially high defensive response as the skin and lungs may have been an important factor in these results.

Although most of the worms found were mature, the majority of the inocula, however, were not recovered. Did those that were lost die in the lumen? Were they passed as live larvae that failed to penetrate the mucosa? Lack of penetration would be at least one logical explanation, inasmuch as there was not nearly enough evidence of dead larvae or areas of reaction in the intestinal wall to account for those that failed to develop. In fact, the evidence points to the likelihood that most of the larvae that penetrated into the gut wall were able to complete their development. Perhaps, too, the worms tended to be eliminated quickly when they reached maturity in the lumen.

The rate at which gut contents are eliminated, the nature of the contents, and other factors such as the penetrability of the mucosa itself, may have been more important to the success of the infection with this parasite than the reaction that occurred once the larvae invaded the tissues. Further studies on the dynamics of the infection are needed.

Of considerable interest, also, are the findings with the mouse tapeworm, *H. nana.* Larsh (1945, 1951) has summarized the information relating to *H. nana* in abnormal hosts, and has discussed some of the factors involved in natural resistance to this cestode. Two previous reports in the literature ndicate that *H. nana* grows only poorly or not at all in the conventional guinea pig. Ransom (1921) found that a guinea pig kept with some rats contained everal immature forms of *H. nana*, presumably acquired from its companions. The largest of the parasites was only about 3 mm. in length, but had 50 to 60 segments. Genital primordia were present. Hunninen (1935) infected 9 one-month-old guinea pigs with 5000 eggs each. Four per cent of the eggs were recovered as cysticercoids in 13 of the animals necropsied at 93 hours, ut no worms were found in the remaining 6 on day 15.

In our experiments the length of the prepatent period in the germfree guinea ig fell within normal limits. In experiment No. 1, mature tapeworms and eggs free in the feces were found at necropsy on day 17 while, in experiment No. 3, only immature forms were found on day 11. Shorb (1933) demonstrated that the prepatent period in the mouse was 15 days, which is longer than in the rat. He showed, in addition, that the size range of *H. nana* may be considerable. It would appear that the worms recovered from the germfree guinea pigs would fall at the lower end of the range he presented.

Aside from the fact that this cestode parasite was more successful in the germfree than in the conventional abnormal host, there are interesting implications with regard to the physiology and nutrition of this tapeworm. Inasmuch as this parasite does not have a gut and absorbs its food from the contents of the lumen, the results point to the apparent lack of a need for products of the metabolism of intestinal flora. These findings could open up new appraoches to the study of the nutrition of cestodes and to their *in vitro* cultivation.

Summary

The rat nematode *Nippostrongylus muris*, the mouse nematode *Nematospiroides dubius*, and the mouse tapeworm *Hymenolepis nana* were found to develop to apparently normal fertile adults in germfree guinea pigs, animals generally considered to be abnormal hosts for these parasites. While worm yields in the case of *N. muris* were very low, in the case of *N. dubius* they were as high as 23 per cent of the larvae inoculated. Development of these helminths in conventional guinea pigs was either extremely poor or failed to occur. The significance of these findings is discussed.

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Discussion of the Paper

QUESTION: I believe that it was stated that the rat nematode usually infects by burrowing through the skin, and I believe that the method you employed was subcutaneous inoculation of larval forms. I wonder whether this nematode is capable of penetrating the host by the normal route, that is, through the skin?

NEWTON: Normally, these larvae do penetrate through the skin, but the laboratory method is to inject them subcutaneously so that a known number can be used.

QUESTION: I was wondering whether these parasites, in which a tissue phase exists, can be transmitted through the placenta in the normal host? In other words, is there evidence for placental transfer in the mother rat of these nematodes into the embryo through the uterus?

NEWTON: You probably are referring to the work done with the dog. Certainly, some of these larvae will penetrate the placental barrier; the hookworm will also pass into the embryo by this route.

THE USE OF GERMFREE GUINEA PIGS IN STUDIES ON THE MICROBIAL INTERRELATIONSHIPS IN AMOEBIASIS*

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Microbial interrelationships may be significant in the development of any infectious enteric disease. A variety of microbial species is present in all such infections. Although many intestinal inflammations occur only if a specific microorganism is present, and hence has the etiological responsibility ascribed to it, the potential role of its microbial associates has remained, for the most part, unknown. It is a well-known fact, however, that many individuals harbor, for long periods of time without symptoms, organisms of the so-called pathogenic variety which, in others, produce acute disease. In many of these individuals this seeming immunity to the potential disease-producing agent cannot be rationally explained. A classic example of this phenomenon is *Endamoeba histolytica*, the so-called etiological agent of intestinal amoebiasis.

Intestinal amoebiasis is universally distributed and occurs in all age groups from every economic level. Its manifestations range from asymptomatic infection to acute disease; in the latter instance, the host may even succumb to the infection. Similarly, the pathology of the disease may vary considerably among those affected. In the asymptomatic infection, the pathology, if present, may show from a few to many tiny craterlike lesions usually confined, to the intestinal mucosa. These lesions often show a tendency toward rapid healing. The pathology of the more severe infections varies considerably, but is usually of two major types. First, in the erosive type there is superficial erosion of extensive areas of the mucous membrane with but little tendency of the amoeba to penetrate the deeper tissue layers. In the second, more invasive type of infection, the amoebas, accompanied by bacteria, penetrate deep into the tissue layers and migrate laterally, especially in the submucosa. This causes an undermining of the more superficial tissues, which then tend to slough off into the lumen. In rare instances perforation of the intestinal wall may even occur.

There are many unsolved problems of amoebiasis. These have been discussed brilliantly by Rees (1955), by Meleney (1957), and by Otto (1958). The question as to why *E. histolytica* may live compatibly with the host in some instances and produce acute disease in others has not been completely answered. The marked differences in the pathology of the disease among infected individuals cannot be causally explained. It is known that the pathoenicity of *E. histolytica* may vary considerably among different strains (Mele- ey and Frye, 1933), and it is reasonable to postulate that individuals may vary ı their susceptibility to acute amoebic disease. It is highly questionable, owever, whether these factors alone may explain the variety of host-parasite elationships that accompany amoebic infection. Perhaps the degree of com- atibility existing between the amoeba and the intestinal flora and/or the resence (or absence) of certain bacterial species in the host's intestine may ıfluence the course of the infection. It was to investigate the potential role f bacteria in this disease that we initiated our studies in germfree experimental osts. The results of these investigations, for the most part, have been re- orted elsewhere (Phillips *et al.*, 1955; Phillips, 1957; Phillips *et al.*, 1958). A eview and summary of these data provide the basis for the present report.

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* This paper is a review of studies conducted over a period of nearly six years by ourselves and our associates at the Lobund Institute. These investigations were supported in part by Contract NR: 131-167 between the Office of Naval Research, Department of the Navy, Washington, D. C., and the University of Notre Dame.

Guinea pigs were employed as germfree experimental hosts in all the experi- nents. Methods for obtaining, rearing, inoculating, examining, and deter- nining the microbiological status of the germfree animals have been described Phillips *et al.*, 1955, 1958). All amoebic inocula were derived from amoeba- rypanosome cultures maintained by the method of Phillips and Bartgis (1954). onventional guinea pigs, maintained on the same sterilized dietary regimen iven the germfree animals, were inoculated as controls in some experiments.

In the first series of experiments, 73 germfree guinea pigs were inoculated ıtracecally with *E. histolytica* and sacrificed at intervals of 1 to 58 days fol- owing inoculation. None of these animals had gross lesions at necropsy. Iicroscopic examination of the cecal tissue did not reveal amoebas in any of he tissue layers, and there was no indication that they had invaded the in- estinal epithelium in any instance. There appeared to have been no multipli- ation of *E. histolytica* in the lumen of the germfree intestine, and survival of he amoebas therein was limited, seemingly, to but a few days. On the other and, conventional (control) guinea pigs that had received portions of the same ıocula given the germfree animals developed amoebic disease. Fifty-three onventional animals received amoebic inocula, and 41 of these developed cute ulcerative amoebiasis.

These results appeared to have answered the question of primary interest o us in the investigations. This was the question as to whether *E. histolytica* capable of producing, independently of its microbial associates in the in- estine, any or all of the wide and varied range of disease processes attributed o it. It then seemed of interest to evaluate the disease-producing potential f the amoeba in association with various single species of bacteria. We did ot discover whether the apparent dependence of *E. histolytica* on bacteria in- olved multiple bacterial species or only certain species, or whether the amoeba ould survive and produce disease in the host with any of the bacteria more ommon to the lower intestine. To obtain information within this area, germ- ee guinea pigs were contaminated with single species of bacteria prior to noebic inoculation.

In the first experiment an anaerobic Gram-negative bacillus, that is, one nidentified but commonly referred to as the Shaffer-Frye *Streptobacillus* (Shaf- r and Frye, 1948), was employed as the monobacterial agent. The amoeba failed to produce lesions in animals harboring this bacterium, but the results were inconclusive. Although this *Streptobacillus* became established in the intestine of the animals, it grew only at very low, possibly ineffective, levels in any of the animals involved. In two subsequent experiments, *Escherichia coli* and *Aerobacter aerogenes* were employed as the monobacterial agents in separate experiments. Seven animals harboring *E. coli* received amoebic inoculation, and 6 of the animals developed amoebic lesions. Five animals harboring *A. aerogenes* were inoculated with *E. histolytica*, and all developed amoebic lesions. Three of the animals with *E. coli* and 2 with *A. aerogenes* showed extensive areas of ulceration on the cecal wall. In another experiment *Bacillus subtilis* was introduced into germfree guinea pigs intracecally, together with the amoebic inoculum. The experiment involved 8 animals, and 7 of these showed amoebic lesions on the cecal wall when sacrificed. Four of the animals developed an acute ulcerative amoebiasis nearly comparable to that which occurred in the conventional (control) animals.

These results provided additional evidence for the participation of bacteria in the etiology of intestinal amoebiasis. Some unidentified factor or factors contributed by bacteria seemed to be essential for the maintenance of *E. histolytica* in the intestine and, possibly, to the pathogenesis of amoebic disease. Consequently, it appeared desirable to ascertain whether these factors were provided only through the continuing activities of living bacteria or whether they may be adequately present in heat-treated preparations of bacteria and their metabolic end products. To obtain this information the cecal content (suspended in normal saline) from conventional guinea pigs, or the sediment or supernate from centrifuged preparations thereof, was autoclaved into the germfree tanks and fed to 60 germfree guinea pigs for 2 days prior to and for 2 days following amoebic inoculation. Twenty-one of these animals developed an amoebic lesion on the cecal wall at the site of amoebic inoculation. Active amoebas were observed in scrapings of the lesions in all but one of these animals. The shortest postinoculation period wherein a lesion containing active amoebas was observed was 7 days. The oldest lesion containing active amoebas was observed on the sixty-third postinoculation day.

In related experiments, autoclaved filtrates of preparations of cecal content from conventional animals were administered to germfree guinea pigs intracecally, together with the amoebic inoculum. In most instances the filtrates were prepared with Seitz filters ranging in porosity from 4 to 10 μ. In several instances, however, the filtrates were prepared with filters having a porosity of 0.5 μ. Twenty-nine animals received filtrates prepared with the 4- to 10-μ filters, and 10 of the animals developed a localized lesion containing active amoebas at the site of inoculation through the cecal wall. On the other hand, 27 animals received filtrates prepared with filters having a porosity of 0.5 μ, and none of these developed amoebic lesions.

In other experiments the reducing agents sodium thioglycollate and cysteine HCl, along with the amoebic inoculum, were administered to germfree guinea pigs. Related studies had shown the oxidation-reduction (redox) potential in the cecal lumen of germfree and conventional guinea pigs to be markedly issimilar. The average (redox) potential in germfree animals was approxi- nately −90 mv., whereas in conventional animals the potential averaged ap- roximately −367 mv. A total of 27 germfree guinea pigs received either of he 2 chemical preparations along with the amoebic inoculum, and 15 of them leveloped a localized lesion on the cecal wall at the site of inoculation. In 11 f the animals, active amoebas were observed in scrapings of the affected areas. Twelve animals that received intracecal injections of the chemical, and *Try- anosoma cruzi* but no amoebas, remained free from any detectable lesions.

In the final experiments reviewed here, the cecal tissue in germfree animals vas severely traumatized at the time of amoebic inoculation. The trauma was roduced by repeated needle puncture in some instances, and by scraping the ecal epithelium with the point of a hypodermic needle in others. The trau- natized areas were approximately 20 mm. in diameter and surrounded the site f amoebic inoculation. Twelve germfree animals were thus treated at the ime of inoculation, and 4 of these developed a localized amoebic lesion within he area of the traumatized tissue. Active amoebas were found in scrapings f the lesions in 3 of the animals.

Some incidental results showed that the cultural stages of *T. cruzi*, which vere present in all amoebic inocula, survived for periods of only 6 to 36 hours n the intestinal lumen of germfree and conventional guinea pigs; trypanosome nfection of tissue did occur, however, in germfree animals. Almost all germ- ree guinea pigs sacrificed 2 weeks or more following inoculation showed a arasitemia with *T. cruzi*. In a limited number of animals examined by ther histopathological procedures, so-called pseudocysts containing *Leishmania* tages of *T. cruzi* were observed within the muscle fibers of the myocardium. Similar examination of blood and other tissue from conventional guinea pigs resented no evidence of *T. cruzi* infection, although these infections are known o occur in conventional guinea pigs following intracecal inoculation with cul- ural stages of *T. cruzi* (E. J. Tobie, National Institute of Allergy and Infectious Diseases, unpublished data). The demonstration of trypanosome forms was one only upon direct examination of circulating blood; admittedly, more laborate diagnostic procedures might have demonstrated *T. cruzi* in our con- entional animals. However, numerous trypanosomes were observed in the lood of germfree guinea pigs by procedures that failed to reveal any of the rganisms in conventional animals that received similar inocula. Conse- uently it would appear that although the germfree guinea pig, unlike the con- entional animal, is unsusceptible to amoebic infection, its susceptibility to nfection with *T. cruzi* may surpass that of its conventional counterpart.

TABLE 1 summarizes the results of these investigations. These data show hat, when *E. histolytica* was introduced into conventional guinea pigs, acute lcerative amoebiasis was produced. A similar disease occurred following ntroduction of the amoeba into guinea pigs harboring *B. subtilis* as their only acterium. A more moderate form of the disease developed when the amoeba vas introduced into guinea pigs harboring either *E. coli* or *A. aerogenes* as their nly bacterium. Conventional guinea pigs and those harboring single bac- erial species that did not receive amoebic inoculation all remained free from

TABLE 1

EFFECTS OF *ENDAMOEBA HISTOLYTICA* ALONE, WITH CERTAIN BACTERIA, AND WITH BACTERIAL OR CHEMICAL PREPARATIONS, ON GERMFREE AND CONVENTIONAL GUINEA PIGS (All Amoebic Inocula Derived from Cultures of *E. histolytica* and *T. cruzi*)

Guinea pig	Inoculum (Intracecal)		Results
Germfree	+ *E. histolytica*		No lesions
Conventional	+ *E. histolytica*		Extensive ulceration
Germfree	+ *E. histolytica*	+ *B. subtilis*	Extensive ulceration
Germfree	+ *E. histolytica*	+ *A. aerogenes*	Moderate ulceration
Germfree	+ *E. histolytica*	+ *E. coli*	Moderate ulceration
Germfree	*None*	+ *E. coli*	No lesions
Germfree	+ *E. histolytica*	+ Autoclaved cecal content*	Amoebic abscess at site of inoculation
Germfree	+ *E. histolytica*	+ Autoclaved filtrate of cecal content†	Amoebic abscess at site of inoculation
Germfree	+ *E. histolytica*	+ Autoclaved filtrate of cecal content‡	No lesions
Germfree	+ *E. histolytica*	+ Chemical reducing agent	Amoebic abscess at site of inoculation

* From conventional guinea pigs and administered orally.
† From conventional guinea pigs and given with inoculum (filter porosity 4–10 μ).
‡ From conventional guinea pigs and given with inoculum (filter porosity 0.5 μ).

any similar disease. Although the causal relationship of *E. histolytica* to the lesions produced was thus demonstrated, the variations in the disease among the several experimental groups suggested that the extent and type of lesions that develop in intestinal amoebiasis may depend upon which species of bacteria are present.

The failure of *E. histolytica* either to produce lesions or to survive when introduced intracecally into germfree guinea pigs is of particular interest. It is significant, perhaps, that these results are similar to those obtained in attempts to cultivate the amoeba in the absence of associated cells *in vitro*. Subsequent investigations, it is hoped, may provide methods that will permit the amoeba to grow in the intestinal lumen of germfree hosts. Only under these conditions would it be possible to obtain unequivocal information concerning the inherent disease-producing potential of *E. histolytica*. Under the conditions of our experimentation, bacteria were required to provide factors essential to establishing amoebic infection in the intestinal lumen; perhaps this may be the most fundamental contribution of bacteria to the development of amoebic disease.

The occurrence of localized amoebic lesions in germfree guinea pigs that had received preparations of conventional cecal content and certain chemical supplements to the diet or to the amoebic inoculum cannot be causally explained. The lesions occurred at the sites of inoculation and involved areas 5 to 40 mm. in diameter. There was no indication that any of the preparations extended the survival time of amoebas in the intestinal lumen. Even when cecal lesions containing active amoebas were observed, the organisms were not found in the cecal content. It is known, however, that chemical reducing agents of the type employed in the studies are favorable to survival of the amoebas *in vitro*. Furthermore, it is not improbable that some factor or factors favorable to the moeba were present in the preparations of conventional cecal content. Con- equently, it is possible that some factor may have been provided that tended preserve the vigor of the amoeba, possibly for only a short time, thus en- ancing its chances for coming in contact with, and becoming established in, e tissue damaged by injection of the inoculum through the cecal wall.

When *E. histolytica* became established in germfree tissue under the condi- ons described, it grew therein for prolonged periods of time in the complete bsence of any bacterial influence. Active amoebas were found in scrapings localized cecal lesions in germfree guinea pigs for periods as long as 63 days llowing amoebic inoculation. Even in these instances, however, the lesions ore no resemblance to those that develop in the presence of bacteria in the onventional host. Extensive ulceration, which characterizes the disease in e conventional animal, did not occur; instead, the lesions were of a nodular pearance and were located, for the greatest part, on the exterior of the cecal all. The inflammatory reaction was atypical and limited almost exclusively increased proliferation of fibroblasts and the deposition of fibrin, although few lymphocytes were present in the area involved. In all instances, the nall foci of infection were walled off by the tissue response of the host organism.

Intestinal amoebiasis, as we know it in the conventional host, was not roducible in the germfree guinea pig. *E. histolytica*, when disassociated from cteria in the germfree intestine, showed no tendency toward the production lesions in normal tissue. When the amoeba was associated with each of ree known species of bacteria, however, ulcerative lesions were produced. onsequently, it has been demonstrated that, although a given microbe alone ay be completely harmless to its animal host, this same microbe, when as- ciated with other equally harmless microorganisms, may produce a disease such severity as to jeopardize the life of the host. Intestinal amoebiasis is striking example of the fact that microbial interrelationships can be involved sentially in the etiology of enteric disease.

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Discussion of the Paper

QUESTION: I should like to ask if the animals monocontaminated with *Escherichia coli* or *Aerobacter aerogenes* had ceca smaller than the germfree animals?

PHILLIPS: I cannot give a definitive answer to this question; we were interested, primarily, in whether amoebic lesions were present in the animals. It is my impression, however, that cecal distension was less pronounced in the monocontaminated animals even though they did not show conventional characteristics in this regard.

QUESTION: Do you believe that certain antibiotics effect a cure in intestinal amoebiasis indirectly by inhibiting the bacteria of the intestine?

PHILLIPS: I can only speculate on an answer to this question. In the work we have just reported it was shown that *Endamoeba histolytica*, once it gained access to germfree tissue, survived therein for long periods in the complete absence of other microbial influence. For this reason it would seem improbable that a cure of acute intestinal amoebiasis could be effected indirectly by suppressing the activities of intestinal bacteria. Although I cannot give a precise answer to your question, I believe data currently available suggest that bactericidal but nonamoebicidal drugs such as penicillin provide cures of the symptoms (only) in some cases of acute intestinal amoebiasis by their suppression of bacterial activity. A cure of the amoebic infestation is not concurrent, however, and recurrences are the rule. The most successful antibiotics employed in the treatment of intestinal amoebiasis are those that have shown a high direct amoebicidal potential.

HEMORRHAGIC SHOCK IN GERMFREE RATS[*]

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The presence of potentially pathogenic bacteria in the gastrointestinal tract
id in the lungs creates no hazard under normal circumstances, chiefly because
iese tissues possess defense mechanisms that actively prevent the dissemina-
on of bacteria or their products. Animals subjected to various forms of stress
iow a progressive deterioration that terminates in the collapse of the peripheral
rculation and fatal shock. With protracted stress, the resulting shock be-
›mes increasingly difficult to treat by conventional therapeutic measures.
fatal outcome is associated with extensive histopathology in the liver and
testinal tract, together with numerous biochemical changes in the blood.[1]
he concept has been advanced[2, 3] that perhaps the most important single fac-
›r contributing to the irreversible trend of the shock syndrome is the inter-
ention of bacteria and bacterial products. None of the experiments on con-
entional laboratory animals has been able to resolve this issue because of the
ultiplicity of uncontrolled factors involved.

The experimental model used most frequently to study the irreversible shock
ndrome is that produced by graded hemorrhage.[4] Fine and his co-workers[2]
und that they could circumvent the irreversible features of the syndrome by
etreating their animals with antibiotics. The most effective form of pre-
eatment was oral, indicating to these investigators that bacterial elements
enteric origin were the important factors contributing to the breakdown of
ie circulation following severe hemorrhage. They also found[5] that the ir-
versible state was associated with the appearance in the blood stream of a
›xic factor the properties of which resembled those of bacterial endotoxins.
general, their work led them to suggest that severe stress results in a break-
›wn of bacterial defense mechanisms and the subsequent liberation into the
ood stream of bacterial endotoxins, which then interfere with the vascular
ljustments essential for compensation during the shock episode. The bac-
rial hypothesis became all the more appealing when it was shown[6] that bac-
rial endotoxins could by themselves bring about a state of shock that closely
sembled that induced by hemorrhage or trauma. Although the evidence is
ghly suggestive of a causal relationship, findings in other animals do not sup-
›rt such a broad generalization.[7]

A fruitful approach to this problem would be the use of animals reared in a
icteria-free environment. If the bacterial thesis were correct, these animals,
ecause of the absence of bacteria or their products in critical tissues, such as

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my, Washington, D. C.

† Established Investigator of the American Heart Association, Inc., New York, N. Y.

the liver and bowel, should be more resistant to the deleterious sequelae of the sydrome, just as were animals pretreated with antibiotics. A series of experiments was therefore set up in collaboration with Gordon and Reyniers at the Lobund Institute, University of Notre Dame, Notre Dame, Ind., using rats reared in standard germfree housing units.[8] In order to avoid the problem of bacterial contamination, a special plastic hood was designed by Philip Trexler, also of the Lobund Institute, into which germfree animals could be transferred, together with the necessary instruments for carrying out such experiments.

Hemorrhagic shock was used as the experimental condition, since such studies would provide a variety of end points with respect to the hypotensive episode, including mortality figures, capacity to withstand blood loss, response to transfusion, and the usual histopathology.[5] By varying the duration and the severity of the hypotension, it was possible to produce a syndrome that was either reversible or irreversible in ordinary laboratory rats. Inasmuch as animals from different colonies varied in their susceptibility to shock, it was necessary to provide adequate controls. In the present experiments the controls were animals of the Holtzman and Lobund strains that had been placed on the same rigorously processed diet as the germfree rats.

The germfree animals were anesthetized with pentobarbital intramuscularly (3.0 mg./100 gm. body weight) and a cannula inserted into the carotid artery for blood pressure recording and bleeding. The cannula was connected to a calibrated reservoir system that could be adjusted to maintain the blood pressure at a desired level. The animals were transfused after various periods of hypotension, and survival statistics were recorded at 24 hours.

Inasmuch as it was anticipated that germfree animals might be more resistant to shock than conventional ones, the initial studies were carried out with a drastic form of hemorrhagic hypotension lethal to about 70 to 80 per cent of the controls. Included in this series of experiments was a group of rats (listed as monocontaminated) that had been accidentally infected with a slowly growing pleomorphic organism, apparently with no effect on the germfree characteristics of the group. As shown in FIGURE 1, there were no significant differences in the responses of germfree and conventional rats to this procedure. The total blood loss, the decompensatory tendency (as manifested by take-up from the reservoir), the blood pressure response to transfusion, as well as the ultimate survival, were essentially the same in both groups (TABLE 1). At autopsy, one finding made it necessary to consider carefully the morphologic background of the germfree animals. As has been reported elsewhere, the cecum of the germfree rat is considerably enlarged and thin-walled.[9] Following shock, several of the animals showed extensive hemorrhage into the cecum. This unusual circumstance, however, was not encountered consistently in all experiments. Actually, it was found that some of the germfree rats that did not have an enlarged cecum showed no such local hemorrhagic involvement and were equally susceptible to hemorrhagic shock. Careful evaluation leads us to believe that this factor is not a major determinant of the irreversibility in germfree animals and thus cannot be used to explain any unusual susceptibility to hemorrhage.

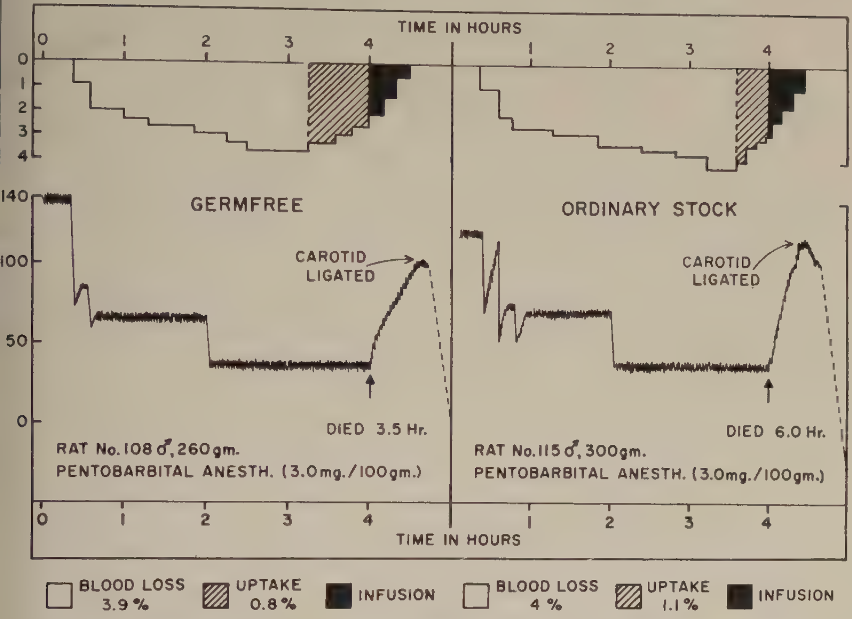

FIGURE 1. Representative protocols in graph form of shock experiments carried out der identical circumstances in germfree and conventional rats. Both animals were on the ne diet (No. 356, a semisynthetic ration). After being kept at the pressure levels indicated 3 hours, the blood remaining in the reservoir was reinfused (indicated by arrow) and the ck incision closed. There were no significant differences in the response to graded hemorage, except that the blood pressure in the germfree rat showed no tendency to rebound er the initial 1 per cent and 2 per cent bleedings, as did the pressure in the controls.

TABLE 1

HEMORRHAGIC SHOCK IN RATS REARED IN A BACTERIA-FREE ENVIRONMENT

Animal	Experimental environment	B.P. during shock	No. of rats	Blood Loss	Blood Up-take	Fate
		(mm. Hg)*		(percentages of body wt.)		
rmfree‡	Open	50–35–35	3	4.0	1.4	3/3 D†
rmfree‡	Sterile hood	65–40–40	5	3.7	0.8	2/5 D
onocontaminated	Sterile hood	54–35–35	7	2.4	0.6	7/7 D
nventional‡	Open	50–35–35	6	3.4	0.9	5/5 D
nventional	Sterile hood	45–35–35	5	3.2	0.6	5/5 D
nventional	Sterile hood	65–40–40	5	3.5	0.7	2/5 D
monocontaminated	Open	45–35–35	2	2.8	0.8	0/2 D

* Three hours successively at blood pressure indicated.
† Fraction indicates number of deaths in numerator and total number in denominator.
‡ All of the animals were on a fortified diet except those indicated with (‡), which were a semisynthetic diet (for details see Zweifach *et al.*[7]).

TABLE 2

BACTERIOLOGICAL FINDINGS FOLLOWING HEMORRHAGIC SHOCK

Animal	Experimental environment	B.P. during shock	Contamination
		(mm. Hg)	
Germfree	Sterile hood	65–40–40	None (3)
Monocontaminated	Sterile hood	45–35–35	None (7)
Conventional	Open	50–35–35	2/3*
Conventional	Sterile hood	45–35–35	None (5)
Exmonocontaminated	Open	45–35–35	2/2*

* Samples were taken of blood, lung, peritoneum, spleen, liver, and cecum. Most frequent contaminants were *Streptococcus fecalis, Micrococcus pyrogenes* (*aureus*), *Escherichia intermedium*, and *Aerobacter aerogenes.*

In view of the failure to demonstrate bacterial contamination in cultures of blood or tissues (TABLE 2), the circulatory collapse following shock appears to have its origin in some mechanisms not directly related to bacteremia per se. It was felt that it would be of some interest to infect germfree animals and then to ascertain whether or not this circumstance altered their response to shock. Several animals were therefore removed from their germfree housing units into the open laboratory and subjected to hemorrhage under aseptic conditions. The experiments were carried out immediately after removal from the germfree unit and again 24 hours later. Here again, no differences were encountered in the response to hemorrhage, despite the fact that these animals were found to show a widespread bacteremia (TABLE 2). It is of interest to note that several of the animals, namely, those having the most extensive bacteremia, survived the procedure, whereas two others that had only minimal bacterial contamination succumbed.

Consideration was also given to the possibility that the germfree animals might be more susceptible to shock than the laboratory controls. This circumstance was tested by subjecting rats reared under germfree precautions to a relatively nonlethal period of hypotension. The finding indicates that the germfree rat was neither more nor less susceptible to hemorrhagic hypotension than the conventional animal. It is of some interest to note that studies carried out an two germfree rats in which the microcirculation was kept under observation throughout revealed the same basic pattern of response as in conventional controls.

In terms of pathology, the liver of some of the germfree animals showed an appearance somewhat different from that encountered in controls. In normal laboratory animals the liver was dark, congested, and distended following blood replacement during the irreversible phase of shock. In the majority of the germfree animals the liver appeared small, contracted, and somewhat pale. Histological examination showed no unusual deposits of glycogen or fat that could account for the unusual pallor of the liver. The incidence of hemorrhage and mucosal damage was the same in the gastrointestinal tract of both sets of animals. This finding is at variance with the situation in conventional animals pretreated with large amounts of antibiotics, where shock was not accompanied by hemorrhagic infiltration of the wall or lumen of the bowel.

The suggestion has been advanced that the germfree animals, although reared under bacteria-free conditions, had access to bacterial products (particularly endotoxins) introduced with their food. The lethal outcome of the shock syndrome in the germfree rats would be explained on this basis by the invasion of enteric endotoxins into the blood stream. Several diets were used in each of the experimental categories. The number of dead bacteria in the semisynthetic diet was much smaller than in the fortified diet. However, there were no significant differences that could be attributed to the diets per se.

The present study clearly demonstrates the fact that irreversible shock can be induced in the absence of bacterial factors. The experiments with germfree animals, however, must be interpreted with considerable caution, since the absence of bacteria in their environment is known to be associated with a variety of structural and functional changes. The dietary background also represents another intangible that cannot be weighed satisfactorily in the conventional rat. Inasmuch as the collapse of the circulation appears to be associated with the same basic elements in both sets of animals, the contribution of bacterial contamination to the irreversible trend would seem, at most, to be secondary in nature. The question as to whether there is a release of tissue products[10] similar in nature and action to the bacterial lipopolysaccharides remains unresolved.

The present findings are therefore interpreted as evidence against the primary contribution of bacterial products to the circulatory collapse following stress. The unique value of the germfree animal to studies of this type is self evident.

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Discussion of the Paper

GEORG SPRINGER (*Hospital of the University of Pennsylvania, Philadelphia, Pa.*): According to Landy and Shear,[1] the mammalian host contains material n its tissues that, when separated from its natural environment, is capable of voking many biological phenomena hitherto ascribed to bacterial endotoxins nly. Thus, the question of whether or not bacterial endotoxins contribute materially to the symptoms of traumatic shock might not be answerable by means of the experimental methods used by Zweifach.

ZWEIFACH: To date, no one has demonstrated that tissues removed from ermfree animals contain polysaccharides similar to those fractionated from he tissues of conventional animals. In view of the protracted incubation proedure required to produce such materials, there still remains the question whether or not some contamination may not be involved in their production from conventional tissues. I think that the experiments with germfree animals clearly indicate that a bacterial factor, per se, is not essential for the production of irreversible shock.

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ROLE OF THE INTESTINAL FLORA IN THE GROWTH RESPONSE OF CHICKS TO DIETARY PENICILLIN[*]

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The mode of action by which antibiotics mixed with food cause a growth stimulation in chicks, turkeys, and certain other animals has not yet been defined. Since most evidence suggests that this growth-promoting effect is mediated by the action of the antibiotics on the intestinal flora, studies on germfree birds and birds with a defined flora appear to provide the most direct approach to the problem.

In a colloquium held at the University of Notre Dame, Notre Dame, Ind., in 1952, Luckey[1] reported that no growth stimulation occurred when germfree birds were fed antibiotics at the level of 50 mg./kg. of diet. In a later publication, Luckey *et al.*[2] reported that slightly lower levels of some of the antibiotics (oxytetracycline, 25 mg./kg.; procaine penicillin, 11 mg./kg.) mixed with the diet caused "slight growth increments" in germfree chicks. The number of germfree birds used in these studies was small, however, and no definitive conclusions could be drawn. Recently Gordon *et al.*[3] have repeated some of this work and have concluded that procaine penicillin at the level of 50 mg./kg. diet does not affect the growth rate of germfree chicks. Our results over the past two and one-half years also show conclusively that penicillin has no effect on the growth rate of germfree chicks.[5]

Our studies concern first, the effect of the intestinal flora on the growth of chicks; second, the effect of certain species of bacteria on the growth of chicks ree of all other bacterial organisms; and, third, the growth response to dietary penicillin of germfree chicks, conventional chicks, and chicks with a defined ntestinal flora.

Chickens and Rearing Conditions

White Leghorn chicks were used in these studies. The germfree[‖] chicks were maintained in Reyniers Germfree Units. The method used for rearing germfree chicks was patterned on that of Reyniers *et al.*[4] and is described in detail elsewhere.[5] Conventional chicks were reared either in Reyniers units that were intentionally contaminated or in standard brooders in the animal room. A defined intestinal flora was implanted in chicks by adding bacterial cells of an 18-hour culture to the first rations fed to germfree chicks or by introducing, on the first day of life, an aliquot of a cell suspension into the crop of

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[‖] In this report the word "germfree" is used in the sense of "free of bacteria, fungi, and rotozoa."

each chick.[6] After hatching, the germfree and conventional chicks were subdivided into 2 groups of 7 to 10 each, one group serving as control, the other maintained on the diet supplemented with penicillin.

Diet

A semisynthetic casein-starch diet was used except where specifically mentioned otherwise. The composition of the diets and the sterilization of the food are described in detail elsewhere.[5] Penicillin was sealed in ampules, sterilized by irradiation,* introduced into the germfree units by means of a germicidal trap, and then mixed inside the units with weighed quantities of food. Food and water were offered ad libitum.

Bacteriology

The procedures and methods used to determine the sterility inside the units are described elsewhere.[5] At the termination of some of the experiments, bacterial counts were made in various segments of the intestine of the chicks with a defined flora. The procedure is described elsewhere.[6] The strains of *Clostridium welchii*, *Streptococcus liquefaciens*, *Lactobacillus lactis*, and *Escherichia coli* used in the experiments with a defined flora were isolated from the intestinal contents of chicks reared at the National Institute for Research in Dairying. One other strain of *E. coli* (TABLE 5, below) isolated from the intestinal contents of chicks raised at the University of Maryland, College Park, Md., and a laboratory strain of *Clostridium bifermentans* were also used. The suspensions of bacteria in sealed ampules were introduced into the germfree units by means of a germicidal trap.

Results

The results given in TABLE 1 show that conditions in the Reyniers units do not affect the growth of chicks. Chicks reared in units that were not maintained sterile grew at the same rate as birds kept in brooders in the animal room. The germfree chicks, however, grew at a faster rate than did the conventional birds. These results demonstrate that one or more components of the intestinal flora have a growth-depressing effect. Food-intake measurements suggest that the more rapid growth of the germfree birds is not due to a greater efficiency in food utilization, but rather that the germfree birds simply eat more.[5] The results shown in TABLE 1 also demonstrate that penicillin had no growth-promoting effect on either the germfree or conventional birds. In repeated experiments with various diets over a period of 2 years, no growth response to penicillin occurred in our animal room.[5] This animal room housed only chicks from eggs that had undergone chemical sterilization treatment. Failure to obtain a growth response to penicillin in clean premises had first been reported by Coates *et al.*[7] Lyophilized intestinal contents were obtained from chicks in her laboratory† reared in so-called "infected" premises where a growth

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* Irradiation of the penicillin was done through the courtesy of H. Andrews, National Cancer Institute, and J. Hickey, Sanitary Engineering Branch, National Institutes of Health, Bethesda, Md.

† National Institute for Research in Dairying, Shinfield, England.

TABLE 1

ROWTH AND GROWTH RESPONSE TO POTASSIUM PENICILLIN (25 MG./KG. DIET) OF GERMFREE AND CONVENTIONAL CHICKS REARED IN REYNIERS UNITS AND IN THE ANIMAL ROOM (Sixteen Chicks per Group)

Status	Average weight in grams on day 21	
	No Suppl.	Penicillin
ermfree (GF)	250	238
onv. in Reyniers units (R.U.)	225	230
onv. in animal room (A.R.)	221	228

Analysis of Variance

Source of variation	DF	MS	F	p
ain effects				
GF versus (conv. in R.U. + conv. in A.R.)	1	6.984	8.59	<0.001
Conv. in R.U. versus conv. in A.R.	1	129	<1	Not signif.
Pen. versus no pen.	1	1	<1	Not signif.
All interactions	2	1541	1+	Not signif.
rror term	90	813		

esponse to penicillin occurred regularly. Comparison of the growth and rowth response to penicillin of chicks immediately before and after our animal oom was "infected" with the intestinal contents shows that after the infection he growth was depressed and that penicillin counteracted this growth depression (TABLE 2). Penicillin supplements in the diet restored the growth rate of he chicks to the preinfection level. Comparison of the growth response to enicillin of the germfree chicks with that of chicks reared in the infected nimal room indicates that the growth response to penicillin occurs only in the onventional but not in the germfree chicks (TABLE 3). Penicillin supplements n the diet never accelerated the growth rate of conventional chicks to equal hat of the germfree chicks. Lev *et al.*[8] noted that *Cl. welchii* type A was found n chicks from infected premises one day after feeding, but not in those from he "clean" environment. The growth of germfree chicks was therefore compared to that of chicks in which this strain of *Cl. welchii* was implanted but hat were free of all other bacteria. As shown in TABLE 4, the clostridia caused growth depression, which was partially counteracted by penicillin. Other acteria, such as *E. coli* or *Cl. bifermentans*, implanted in chicks free of all other acteria had no growth-depressing effect, and addition of penicillin to the diet aused no growth stimulation (TABLE 5). Similarly, a combination of 3 acteria, *E. coli*, *L. lactis*, and *S. liquefaciens*, implanted in chicks free of all ther bacteria do not depress their growth compared to that of the germfree hicks.[6] However, when *Cl. welchii* was implanted in chicks in addition to hese 3 bacterial species, the growth of the chicks was depressed.[6] Supplements of penicillin in the diet partially counteracted this growth depression ue to the presence of *Cl. welchii*. Bacterial counts performed at the end of e experiment in the intestinal contents of the chicks harboring only *Cl.*

TABLE 2

GROWTH AND GROWTH RESPONSE TO PROCAINE PENICILLIN (45 MG./KG. DIET) OF CHICKS BEFORE AND AFTER "INFECTION" OF THE ANIMAL ROOM
(Thirty Chicks per Group)*

Status	Average weight in grams on day 21		
	No Suppl.	Penicillin	Diff.
Preinfection	230	222	−8
Postinfection	208	235	+27

Analysis of Variance

Source of variance	DF	MS	F	p
Main effects				
Status: pre versus post†	1	629	<1	Not signif.
Treatment: pen. versus no pen.	1	2,536	<1	Not signif.
Interaction				
Status by treatment	1	9,149	10.06	≪0.01
Error term	116	909		

* Weight data selected at random from 3 replicate experiments before and 3 after deliberate infection of the animal room with intestinal contents from chicks reared at the National Institute for Research in Dairying.

† Student's t test on groups receiving no penicillin supplement in the diet—preinfection versus postinfection: $t = 3.02, p = < 0.01$.

TABLE 3

GROWTH AND GROWTH RESPONSE TO PROCAINE PENICILLIN (45 MG./KG. DIET) OF GERMFREE CHICKS AND CONVENTIONAL CHICKS REARED IN THE "INFECTED" ANIMAL ROOM
(Twenty-four Chicks per Group)*

Status	Average weight in grams on day 21		
	No Suppl.	Penicillin	Diff.
Germfree	251	253	+2
Conventional (postinfection)	207	231	+24

Analysis of Variance

Source of variance	DF	MS	F	p
Main effects				
Status: GF versus conv.†	1	25,643	1+	Not signif.
Treatment: pen. versus no pen.	1	3,940	<1	Not signif.
Interaction				
Status by treatment	1	19,632	28.96	≪0.001
Error term	92	678	—	

* Weight data selected at random from 3 replicate experiments.

† Student's t test on groups receiving no penicillin supplement in the diet—germfree versus conventional (postinfection): $t = 5.63, p = < 0.001$.

TABLE 4

ROWTH AND GROWTH RESPONSE TO PROCAINE PENICILLIN (45 MG./KG. DIET) OF GERMFREE CHICKS AND CHICKS WITH AN INTESTINAL FLORA CONSISTING ONLY OF *CL. WELCHII*

(Fourteen Chicks per Group)*

Intestinal flora	Average weight in grams on day 21		
	No Suppl.	Penicillin	Diff.
one (germfree)	257	254	−3
l. welchii†	213	243	+30

Analysis of Variance

Source of variance	DF	MS	F	p
ain effects				
Status: GF versus *Cl. welchii*‡	1	10,423	2.8	Not signif.
Treatment: pen. versus no pen.	1	2,579	<1	Not signif.
nteraction				
Status by treatment	1	3,681	3.70	0.05–.1
rror term	52	993		

* Weight data selected at random from 2 replicate experiments.

† *Cl. welchii* type A were established at the start of the experiment in chicks free of all her bacteria.

‡ Student's t test on groups receiving no penicillin supplement in the diet—germfree versus *l. welchii*: $t = 3.69$, $p = <0.001$.

TABLE 5

ROWTH AND GROWTH RESPONSE TO PROCAINE PENICILLIN (45 MG./KG. DIET) OF GERMFREE CHICKS AND CHICKS WITH AN INTESTINAL FLORA CONSISTING ONLY OF *CL. BIFERMENTANS* OR *E. COLI*

(Seven to Nine Birds per Group)

Intestinal flora	Diet*	Average weight in grams on day 21		
		No suppl.	Penicillin	Diff.
one (germfree)	C-8	233	234	+1
l. bifermentans	C-8	244	245	+1
one (germfree)	C-7	237	237	0
. coli†	C-7	233	241	+8

* C-8 is a soybean-corn meal diet; C-7 is a casein-starch diet.[5]

† *Cl. bifermentans* or *E. coli* were established at the beginning of the experiments in chicks ee of all other bacteria.

elchii, showed that this organism is present in large numbers in the chicks ceiving the basal diet, but that very few are present in the penicillin-fed icks. When other bacteria are present, the number of clostridia is less than the chicks harboring clostridia only.[6] In chicks receiving the penicillin-pplemented diet, the number of clostridia was found to be even more reduced. one experiment, however, the number of clostridia was not found to be ppreciably decreased in the penicillin-fed chicks. In this case an interesting observation originally made by Lev *et al.*[9] was confirmed: lecithinase production of the clostridia isolated from the penicillin-fed chicks was markedly reduced. Upon subculture, these clostridia produced as much lecithinase as the clostridia obtained from the chicks on the basal diet.[6] Reduction in toxin production by clostridia may have the same effect on the host as the elimination of the bacteria.

Discussion

Penicillin supplements in the diet had no effect on the growth of conventional chicks until the animal room was deliberately infected. This infection caused a growth depression that was counteracted by penicillin. The results of these experiments support the thesis of Coates *et al.*[7] that one of the mechanisms of the growth-promoting effect of penicillin in chicks consists in the reversal of a transmissible growth depression condition. The failure of penicillin to accelerate the growth rate of conventional to equal that of the germfree chicks reveals the existence of other growth-depressing factors not amenable to penicillin treatment.

Cl. welchii, whether present as the sole species of bacteria in the intestines of chicks or as a component of a flora consisting of a total of four species of bacteria, caused a growth depression. This growth depression was to some extent counteracted by supplements of penicillin in the diet. It must be noted, however, that the clostridia in the chicks with a defined and limited flora are present in larger numbers than in normal chicks.

Summary

Comparison of the growth of germfree and conventionally reared chicks demonstrates that on adequate diets one or more components of the intestinal flora inhibit growth.

Certain organisms commonly found in the chick's gut (*E. coli*, *L. lactis*, and *S. liquefaciens*), when implanted in chicks free of all other bacteria, do not depress their growth. However, *Cl. welchii* type A, another component of the intestinal flora, was found to depress the growth rate of otherwise germfree chicks. Penicillin in the diet largely overcame the growth depression caused by *Cl. welchii*. Thus penicillin, which had no effect on the growth of germfree chicks or on that of conventional chicks reared in "clean" quarters, was shown to stimulate the growth of chicks with a defined intestinal flora by inhibiting bacteria that retarded growth.

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TUMORIGENISIS AND THE GERMFREE CHICKEN: A PRELIMINARY REPORT*

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Introduction

The use of germfree animals in cancer research is relatively new.[1] This communication considers briefly some of the possibilities and problems inherent in such an approach.

A germfree animal is one that is free from living contaminants.[2] Obviously, this depends upon how a contaminant is defined and upon the techniques available for detecting it. The fact that the definition has these practical limits does not change it. A germfree animal must, by definition, be free from living contaminants; if a contaminant can be detected, the animal is not germfree. It may be considered a *gnotobiote* (*gnos*—known; *bios*—life)[3] since it is associated with a known contaminant. As such, its value may be of equal or greater importance than if it were germfree. Absolute freedom from contamination for an animal may be a theoretical goal, but nevertheless it is an important one to investigate.

At present chickens can be obtained free from bacteria, yeasts, fungi, protozoa, endoparasites, ectoparasites, and many disease-causing viruses.[4] They can be reared germfree to maturity and at least into one generation.[5] Whether germfree chickens can also be obtained free from latent or cytopathogenic viruses is not yet known; first, because the subject has not been investigated in germfree animals and, second, because such tests are difficult to make on a living animal and are presently not available for inclusion in routine testing programs. However, it is the investigation of these not-too-well-defined areas that offer so much to cancer research.

In the final analysis, what constitutes a living being is a matter of definition. At the moment it does not matter whether cell particulates are or are not called viruses as long as they can be isolated, examined, and defined; neither does it matter whether they are considered as contaminants. These are matters that can be decided at a later date when more information is available.

In considering germfree animals for cancer research we were motivated by two main objectives: first, the possibility that subcellular particles might be related to tumorigenesis and, second, the desire to study immunity to tumor formation. The germfree animal, being free from contamination, constitutes a "pure" animal.[6] Because it has never had experience with microbes, it may be considered as "immunologically immature" in the sense that certain defensive systems with which it is endowed by nature to protect it from microbial assault are not activated at birth. On this basis it is possible to apply the pure-culture concept to the search for etiological entities, secure in the knowledge that no contaminants will be added from the environment and that the animal

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has not been conditioned by microbial experience. Even if it is eventually demonstrated that living agents are present as latent viruses or as particles that are inherently components of normal cells, they would be "locked" into a situation and at a level more amenable to investigation than is the case with conventional life.

For the general approach to tumorigenesis using germfree animals, three species have been studied: the albino rat, the inbred C3H mouse, the inbred white Swiss mouse, and the chicken. Rats and mice are included because they can be reared germfree through many generations and can be held to the limits of their lives. The chicken was selected because of its well-known relationship to tumor-inducing viruses, for example, the Rous sarcoma and lymphomatosis. While this bird can be bred through many generations, its size, its need for cage space, its general problem of care, its longer generation time, and its greater life span made it impractical for the initial studies.

In these studies involving the germfree animal and tumorigenesis, an approach consisting of the following nine steps has been followed: (1) study of the germfree animal in terms of its susceptibility to a tumor-causing virus; (2) consideration of spontaneous tumors in germfree species where they are known to arise in the same species reared conventionally (for example, the inbred C3H mouse); (3) induction of tumors by physical, mechanical, or chemical stimuli; (4) transmission of induced tumors by cell transplants; (5) inactivation of the tumor cell and transmission of the tumor; (6) transmission of tumors by fractions of tumor cells obtained by centrifugation or filtration; (7) concentration and purification of the active cell fractions; (8) characterization of the active cell fractions; and (9) study of tumor immunity or tumor-causing fractions of cells.

The above general pattern of study is given to clarify the experiments reported here. It is clear that each step is dependent upon the findings from the previous step and varies slightly with different species, but the general structure remains the same.

A description of the germfree chicken with respect to its anatomy, development, nutrition, and serology has been given elsewhere.[7] This has been amplified with respect to serology,[8] anatomy and physiology,[9] and biochemistry.[10-13] In comparison to conventionally reared chickens (35 to 37 days of age), the germfree chicken shows a lower weight of the small intestine and cecum, lower weight and lower concentration of lymphoid elements in the ileocecal tonsil, increased lymphocyte concentration in the thymus, and no change in adrenal weight or in ascorbic acid concentration.[14] At this age, antibodies against bacterial antigens are absent, but antibodies can be produced by parenteral injection of antigens.[8]

When a germfree chicken is exposed to a conventional environment (that is, "conventionalized") with a mixed microbial population, if it survives it will, in about two weeks, assume the anatomical and physiological characteristics of the conventional fowl. The impact of contamination brings about rapid changes in the lymphoid elements of the intestinal tract, and antibodies will form.

Finally, for those investigators unfamiliar with germfree work, it should be pointed out that 4 adult Leghorn chickens can be conveniently accommodated at one time in a single germfree isolator such as the Reyniers RSU-100, -200, -300 series that have been used in this work and that I have described elsewhere in these pages. It is possible to house 4 to 8 Bantam fowl in the same space, which is one of the reasons we have used this species where possible. Five such isolators have been used in the experiments with chickens.

Methods and Materials

Source of chickens. Two varieties of chickens have been used in these studies: the White Leghorn and the Rose Comb Black Bantam (Godfrey).

Eggs from Leghorns were obtained through a commercial hatchery (Hi-View Farms, Lakeville, Ind.). These eggs represent a random selection from the poultry farms of this region. Only clean, unwashed eggs were accepted, since soiled eggs are more difficult to sterilize for germfree work.

Eggs of Rose Comb Black Bantams were obtained through the courtesy of Edward F. Godfrey (Mount Hope Poultry Department, Williamstown, Mass.). This flock has been especially bred for survival standards and is a closed flock. The Godfrey Bantams have been used in these studies since 1954. The flock is free of pullorum, and none of the birds have been vaccinated against any disease. Lymphomatosis was present in the chickens used to start the flock, but it has been eliminated. Coccidiosis is present in the flock. The eggs used are exceptionally clean, and their hatchability is about 70 to 80 per cent (Godfrey, personal communications).

At an earlier period we found that small Bantams are quite satisfactory for germfree work.[15] Such fowl can be obtained germfree and reared on autoclaved semisynthetic diets or on practical diets through an entire life cycle and into a succeeding generation.[5]

The Godfrey Bantams are a healthy breed. They grow well, produce eggs of good quality, and are remarkably sturdy animals. For these reasons they merit wider use in research.

Methods for producing germfree chickens. The methods for producing and rearing germfree chickens have been described elsewhere. The chickens used in this work were reared in standard Reyniers Germfree Isolators.

In the series of chickens presently reported the shells were sterilized by submerging them for 3 min. in Tergusson,* a detergent-sanitizer. The detergent is allowed to dry on the shells. The eggs were then incubated in a standard Jamesway† egg incubator for 20 days. Those containing living embryos were selected and placed in a nylon mesh tube that was submerged for 5 min. in a germicidal trap, described elsewhere in this monograph, containing 2 per cent $HgCl_2$. The eggs were then placed in a germfree isolator, where they were removed from the nylon tube and incubated to hatching with the $HgCl_2$ dried on the shell surface. At hatching, the chicks were removed from the vicinity of the shell fragments and placed in wire-mesh cages on a towel. The towel as removed after a few days, and the chickens kept on a wire-mesh floor. en to 15 eggs were placed into the germfree isolator, and about 60 to 70 per ent of them hatched. Other eggs from the same clutch were allowed to hatch the standard incubator and were used as conventional controls where needed.

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* Produced by the Dr. Salisbury Laboratories, South Bend, Ind.

† Product of the Jamesway Manufacturing Co., Ft. Atkinson, Wis.

Testing for contamination. The general routine microbiological tests used to etect contaminants have been described elsewhere.[4] In the present experiients, routine testing has been done twice weekly. Samples of fresh feces and aste consisting of droppings, food, feathers, and water from the cage bottom ere placed in thioglycollate broth* and on blood agar plates that were incuated aerobically and anaerobically. The samples were incubated at room emperatures (37° to 55° C.) for 1 to 2 weeks, during which period they were xamined microscopically. Samples of fresh feces were also examined microcopically in wet mounts and, routinely, as fixed preparations stained by Gram's ethod.

Occasionally, these tests were supplemented with others. When animals ere sacrificed within the germfree isolator, samples of cecal contents, tissues, nd blood were examined both in media and microscopically. Special tests or PPLO have been performed and samples of blood from a number of different xperiments were examined serologically for PPLO (personal communications rom J. F. Crawley, Connought Medical Research Laboratories, Toronto, anada). Flotation tests have also been carried out for parasites. These pecial tests have been consistently negative and thus confirm the routine tests.

On several occasions tumor and normal tissue from germfree chickens have een examined in tissue culture for viruses by Alton R. Taylor of the Virus esearch Division, Parke, Davis & Company, Detroit, Mich. The results have een negative.

Whenever possible, we prefer to use a chicken at least 21 days old in order to inimize the possibility of introducing contamination either on the shells or the egg. Younger chickens have been used because of the needs of an experient, and we have accepted the risk involved.

Germfree apparatus. There is little point in repeating a description of the lder-style isolators that we have used. In later experiments, we have used a ew-type Reyniers Isolator (RSU-500).† This is a larger isolator, 72 inches ng and 34 inches in diameter, especially designed for this work. This isolator IGURE 1) is equipped with an external water tank and has a larger sterile pply lock and external lighting. Two wells have been added to the bottom f the isolator, one in which freezing and thawing may be done; the other is a uick-passage germicidal trap. This isolator is also equipped for filtration and e use of an electric homogenizer (Servall Omnimixer),‡ the cups of which can e chilled during operation of the cold well (FIGURE 2).

Diets and dietary regime. Everything taken into a germfree isolator must e sterilized. This includes water, food, bedding, apparatus and media. Sterilition is done by autoclaving in an attached supply lock at 252° F. for 25 min.

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* Produced by Difco Laboratories, Inc., Detroit, Mich.
† Produced by Reyniers and Son, Chicago, Ill.
‡ Produced by Ivan Sorvall, Inc., Norwalk, Conn.

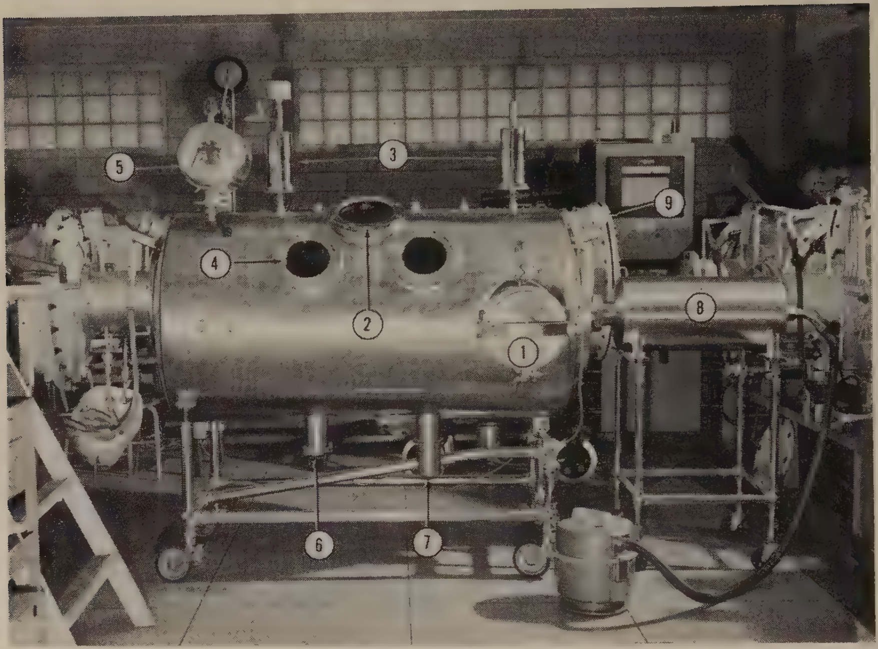

FIGURE 1. RSU-500 germfree isolator. Key: (1) supply lock; (2) one of two viewing windows; (3) inlet and outlet air filters; (4) one of two pairs of gloves; (5) external water sterilizing tank; (6) quick-passage germicidal trap; (7) freezing trap, also used as temperature well; (8) vacuum cleaner for emptying bedding from animal pens; (9) external lamp fitted with ventilating fan and detachable from light window.

The following diets have been used in this work and all were autoclaved, except those used for conventional animals, as noted:

Diet L-291. Purina broiler ration,* 87 gm.; casein Sheffield† N. P., 8.5 gm.; liver (whole Armour‡), 3 gm.; Albimi yeast extract,§ 2 gm.; thiamine, 6 mg.; ascorbic acid, 100 mg.; riboflavin, 2 mg.; calcium pantothenate, 10 mg.; nicotinamide, 10 mg.; choline chloride, 200 mg.; pyridoxine hydrochloride, 2 mg.; *i*-inositol, 100 mg.; biotin, 0.05 mg.; folic acid, 2 mg.; pure vitamin B_{12}, 2 gm.; starch to 1 gm.; total, 100 gm.

Diet L-289. Yellow corn mean, 52.6 per cent; soya meal (solvent) 25 per cent; alfalfa leaf meal, 3 per cent; standard wheat middlings, 10 per cent; fish meal, 2.5 per cent; meat scraps, 3 per cent; salt (iodized), 0.5 per cent; Viadex‖ (4000 I.U. vitamin A, 1000 I.U. vitamin D), 0.25 per cent; calcium carbonate, 1.8 per cent; dicalcium phosphate, 1.6 per cent; manganous sulfate, 12 gm. per 100 lb.; choline chloride, 31 gm. per 100 lb.; thiamine, 90 mg. per 100 lb.; ribo-

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* Product of Purina Mills, Buffalo, N. Y.
† Product of Sealtest Sheffield Farms, New York, N. Y.
‡ Product of Armour & Co., Chicago, Ill.
§ Product of Albimi Laboratory, Inc., Brooklyn, N. Y.
‖ Prepared in our laboratory.

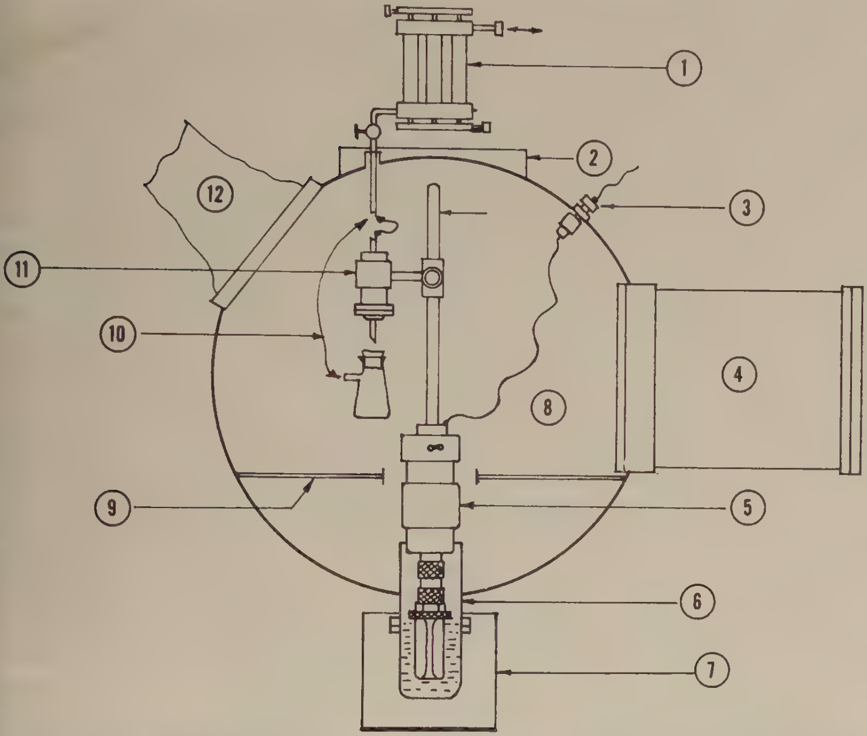

FIGURE 2. Homogenizer and filters inside germfree isolator. Cross section: (1) Reyniers Air Filters; (2) viewing window; (3) electric outlet; (4) supply lock; (5) Servall Omnimixer; (6) temperature well attached to germfree isolator; (7) temperature bath; (8) interior in cross section of isolator; (9) floor of isolator; (10) vacuum flash attached to outlet in wall of isolator; (11) pressure or vacuum filter; (12) rubber gloves.

flavin, 160 mg.; calcium pantothenate, 545 mg.; niacin, 800 mg.; pyrodixine, 160 mg.; casein Sheffield N. P., 70 gm. per kg.

Diet BR. Ralston Purina Broiler Chow,* not antibiotic fortified.

Diet BR/AF. Ralston Purina Broiler Chow, supplemented with antibiotics (purified extractives from *Streptomyces* fermentations and dried *Streptomyces* meals).

These diets and water were fed ad libitum. The lights were turned on in the germfree isolators for approximately 9 hours daily, with some variation for season. Temperatures in the germfree isolators were about 75° F. All diets used yield good growth in both germfree and conventional chickens and are nutritionally adequate for egg production and fertility.

Preparation of the carcinogen. Methylcholanthracene (1.9 per cent) in benzene (MCA/Bz) was put into sealed glass ampules and sterilized by autoclaving. Unless otherwise noted, a single injection of 0.25 ml. of the solution is injected deep into the right breast muscle next to the keel bone.

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* Product of Purina Mill, Buffalo, N.Y.

Benzene is used as the solvent because the solution can be sterilized in accordance with the germfree maintenance routine, and this does not interfere with the carcinogenic action. Methylcholanthrene could not be used in oils because these are difficult to sterilize unless subjected to prolonged heating at high temperatures (180° C.). Single injections of 0.25 ml. benzene into Bantams and Leghorns were tolerated without difficulty.

Preparation of tissue homogenates and filtrates. The tumor tissue homogenates were prepared by cutting the tissue into small pieces that were then minced in a chopper. The finely chopped tissue was then put through a tissue press similar to that of LaPine (FIGURE 3). This tissue was homogenized in either a Waring Blendor* or a Servall Omnimixer 30 per cent v/v saline. In a few instances the homogenized tissue was further reduced in a glass Ten Broeck† grinder or in a mortar with sand.

The Servall Omnimixer may be operated inside the germfree isolator. It is sterilized by sealing the motor in a copper cylinder filled with nitrogen and heating in an oven at 180° C. for 3 hours. The silicone lubricant is sealed in a glass ampule and sterilized at the same time and temperature as the copper cylinder. All cups are completely disassembled. These parts, the copper cylinder, and the ampules of silicone are then autoclaved into the isolator through the attached supply lock. The Omnimixer is reassembled after sterilization and operated inside the germfree isolator.

The Waring Blendor cannot be so sterilized and must be used in the laboratory under aseptic conditions.

The final 30 per cent v/v saline homogenate was used either for injection into the right breast muscle of the chickens or it was further diluted (10 per cent v/v saline) and filtered. The filtrate is then used as a single injection.

Two types of filtering apparatus were used. One was mounted through the outer door of the supply lock and then sterilized and dried in a vacuum (FIGURE 4). This arrangement permits contaminated materials to be filtered directly into the germfree isolator and was used in the experiments on the Rous sarcoma. The second type of filtration apparatus was used inside the germfree isolator with either pressure (nitrogen) or vacuum (FIGURE 2). This system is useful where the operation is confined entirely to the germfree isolator.

In earlier work with the Rous sarcoma the Selas series of filters was used, but they are not as adaptable to working within a germfree isolator as are fritted glass or Seitz filters. A 100 mm. Seitz pressure or vacuum filter was used with sterilizing (St) pads (0.1 μ maximum pore size—mps) and clarifying pads (5 μ mps). The fritted glass filters, 30 and 50 mm. in diameter, were of the following pore sizes: C filter (40 to 60 μ mps); UF filter (0.9 to 1.4 μ mps); M filter (10 to 15 μ mps). Both Seitz and fritted glass filters were first sterilized in the oven and then autoclaved into the germfree isolator by way of the supply lock.

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[*] Produced by Waring Products Corp., New York, N. Y.

[†] Produced by Ace Glass, Inc., Vineland, N. J.

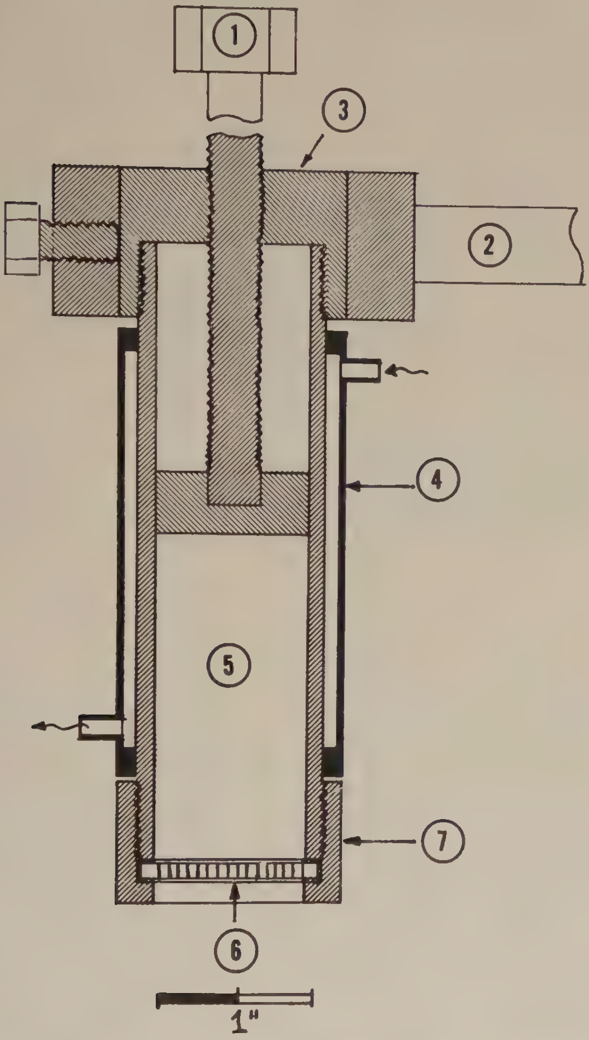

FIGURE 3. Stainless steel tissue press. Cross section: (1) hexagonal nut on finely pitched screw, turned with a ratchet wrench; (2) support fitting over hexagonal head cap; (3) hexagonal head cap; (4) cooling gasket; (5) tissue reservoir; (6) perforated disk fitting against thin gasket; (7) screw cap.

Chicken categories. Leghorn (L) or Rose Comb Black Bantams (B) were used in the following four categories:

(1) *Germfree* (*GF*) chickens in which no contaminants could be demonstrated within the limits of the tests performed.

(2) *Germfree conventionalized* (*GF/CN*) chickens with a history of germfree life during which they were injected, but became contaminated, and were

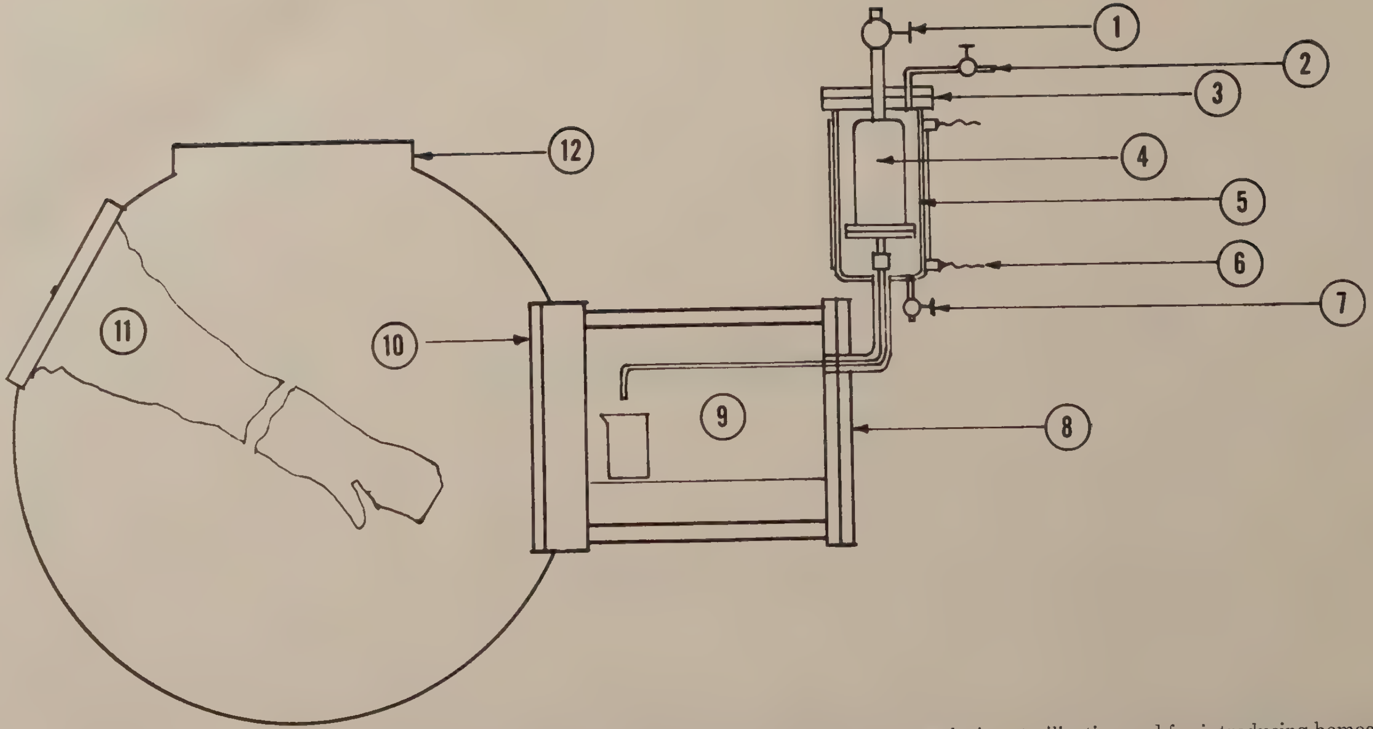

FIGURE 4. Filtering homogenates into germfree isolator. Cross section: (1) relief valve for use during sterilization and for introducing homogenates; (2) relief valve for use during sterilization; (3) lid bolted against gasket; (4) filter; (5) chamber containing filter; (6) cooling jacket; (7) condensate valve; (8) outer door of germfree isolator supply lock; (9) supply lock; (10) inner door of supply lock; (11) gloves; (12) viewing window.

removed to the laboratory chicken rearing room. In this way they were "conventionalized."

(3) *Conventional* (*CN*) chickens hatched from the same clutches of eggs used to produce germfree chickens, but hatched in a standard chicken incubator and reared in a chicken rearing room.

(4) *Antibiotic-fed* (*AF*) or conventional chickens fed from hatch on the diets given elsewhere, to which an antibiotic was added. The level of antibiotic fed was usually 50 mg./kg. of diet, except where noted. Procaine penicillin (P), benzathine penicillin G* (BI), streptomycin (S), and Terramycin (T) were the antibiotics fed in this work.

The prime reason for including the conventional category was to provide greater numbers of animals that could be used for testing the various preparations. The germfree/conventionalized category was included to determine the effect on tumor formation of contaminating the chickens. Antibiotics were fed, not in an effort to study the mechanisms involved, but primarily to obtain a test animal in which the phenomena observed with germfree chickens could be repeated, making it possible to study certain aspects of the problem outside a germfree system.

Experiments and Results

Effect of a known virus (*Rous sarcoma*) *on germfree, conventional, and germfree/conventionalized Leghorn chickens.* The effect of viruses on germfree animals is little known. As a matter of background for these experiments, the Rous sarcoma virus was selected because, in conventional chickens, it will produce tumor following the injection of UF filtrates from the tumor tissue.

The source of the tumor material was a group of conventional Leghorns with Rous sarcoma obtained from C. O. Prickett of the Stine Laboratories.† The tumor was then propagated through homogenates and filtrates in the strain of conventional Leghorns used for the germfree work. In these experiments both the germfree and conventional chickens were maintained on diet L-291.

The filtrates were prepared as follows. Tumor tissue from conventional chickens was cut into small pieces with scissors, minced in a chopper, forced through a tissue press, and ground in a glass Ten Broeck tissue grinder as a 30 per cent v/v suspension in saline. This homogenate was further ground with sand and passed through a C-fritted glass filter. The filtrate was then passed through a UF-fritted glass filter fastened to the supply lock (FIGURE 4) or into glass ampules and sealed. When ampules were used they were then passed through a germicidal trap into the germfree isolator. The UF filtrate was prepared in the same way for both conventional and germfree chickens and was given in a single injection into the right breast muscle, close to the keel bone.

The results of a series of experiments with germfree and conventional Leghorns (TABLE 1) show that UF filtrates from Rous sarcoma tissue grown in conventional chickens will cause a tumor at the site of inoculation. In general,

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* Bicillin, produced by Wyeth Laboratories, Philadelphia, Pa.

† A division of E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

TABLE 1

BACTERIA-FREE FILTRATES OF ROUS SARCOMA INJECTED INTO BREASTS OF GERMFREE AND CONVENTIONAL LEGHORN CHICKENS

Germfree			Conventional		
No. chickens	Tumor incidence per No. injected	Av. time in days to tumor appearance*	No. chickens	Tumor incidence per No. injected	Av. time in days to tumor appearance*
3	2/3	16	3	2/3	19
2	1/2	18	3	2/3	18
2	1/2	25	3	3/3	33
4	3/4	23	3	3/3	32
4	3/4	21	3	2/3	36

N.B. Negative chickens held for 90 days.

* Time to reach 1 cm. in size.

there is a similarity in the over-all picture between germfree and conventional chickens in the tumor structure, the time to the appearance of a 1-cm. tumor, and the subsequent growth and death of the animals.

It is interesting to note that tumor formation in germfree/conventionalized Leghorns appears to be influenced by the experience of contamination (TABLE 2). If the tumor forms before contamination it may continue to grow slowly and become circumscribed; in some instances, it regresses. In the series reported, injections of the UF filtrate into germfree chickens with a history of germfree life up to 23 days postinoculation did not cause tumors, although injections into those held for more than 36 days did so. The conventional controls were positive for tumor formation.

No attempt is made here to quantitate the results, since the prime purpose is to show that germfree Leghorn chickens will respond with tumor formation following the injection of a UF filtrate from conventional Rous sarcoma tissue.

Spontaneous tumors in germfree chickens. The question of spontaneous

TABLE 2

BACTERIA-FREE FILTRATES OF ROUS SARCOMA INJECTED INTO BREASTS OF CONVENTIONAL AND GERMFREE/CONVENTIONALIZED CHICKENS

Germfree/Conventionalized				Conventional		
No. of chickens	Tumor incidence per No. injected	Av. time in days to tumor appearance*	Days of germfree life post-injection	No. of chickens	Tumor incidence per No. injected	Av. time in days to tumor appearance*
3	0/3	—	11	3	3/3	18
3	0/3	—	13	3	2/3	14
6	0/6	—	23	4	3/4	18
3	1/3	18	27	4	2/4	16
4	3/4	25	35	4	3/4	14
3	2/3	17	36	4	4/4	16

N.B. Negative chickens held for 90 days.

* Time to reach 1 cm. in size.

tumors forming in germfree chickens is not of direct importance to the experiments described. Here we are working with a fibrosarcoma induced by MCA/Bz, which appears from 50 to 100 days postinjection and only at the site of injection. This fibrosarcoma is easily transmissible in serial passage from tumor cell homogenates and coarse filtrates in as short a time as 5 days postinjection. Under the circumstances, it would therefore seem that the question of spontaneous tumors would have little bearing on the problem at hand.

However, as a matter of record, it might be noted that we have not seen spontaneous tumors in uninjected germfree chickens held for 400 days.

MCA/Bz-induced tumors in germfree, conventional, and germfree/conventionalized Leghorn and Black Rose Comb Bantam chickens. If a tumor can be induced by nonliving agents, then it is proper to ask if it can be transmitted and, if so, whether this depends upon the tumor cell or some subcellular particulate.

For preliminary experiments MCA/Bz was selected as the carcinogen. It is a well-known carcinogen, and there is an extensive and valuable literature on it encompassing a wide variety of species and experimental conditions.

When MCA/Bz is injected into the right breast muscle of chickens, it will cause a fibrosarcoma. This can be readily observed without sacrificing the chicken, which is an important consideration when numbers are limited. The tumor will form in a large number of cases within 50 to 100 days postinoculation. It grows to large size and in time the animals will succumb to it.

The results obtained from a series of experiments with germfree, conventional, and germfree/conventionalized Leghorn and Bantam chickens are shown in TABLE 3. From these results it can be seen that it is possible to cause a tumor at the site of injection in both varieties of chickens and in the categories mentioned. The toxic effect following injection of MCA/Bz into the Bantam

TABLE 3

MCA/BZ-INDUCED TUMORS IN GERMFREE, CONVENTIONAL, AND GERMFREE/CONVENTIONALIZED LEGHORN AND BANTAM CHICKENS

No. of chickens	Av. age of chickens	Kinds of chickens	No. of chickens with tumor	Time in days to tumor appearance			Terminal age oldest neg. chicken (days)	No. chickens surviving initial MCA/Bz
				Max.	Min.	Av.		
				Germfree				
30	17	Leghorn	26	90	54	68	193	30 30
24	15	Bantam	17	100	75	87	206	24 66
				Conventional				
53	17	Leghorn	51	135	65	92	386	
10	15	Bantam	8	94	50	79	270	10 29
				Germfree/Conventionalized				
39	9	Leghorn	33	125	111	118	365	39 39

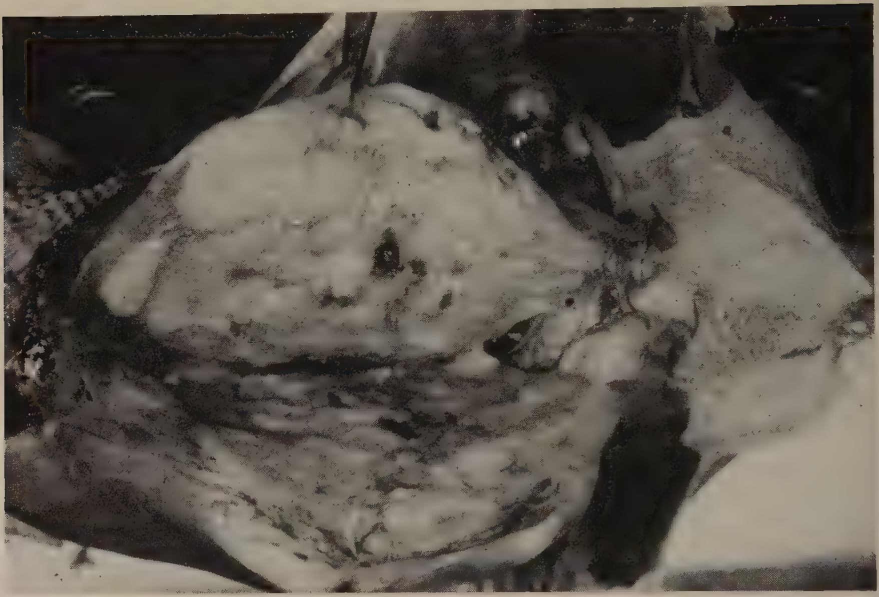

FIGURE 5. Methylcholanthrene-induced tumor in a germfree chicken 60 days after original appearance.

chickens is not due to the benzene alone. Attention is called to the apparently longer time for tumor formation in germfree/conventionalized chickens.

The tumor has been characterized as an adult fibrosarcoma and does not differ in structure or other broad details within each category between the two varieties of chickens (FIGURES 5, 6, and 7).

No attempt is made here to discuss the finer structural details of the tumor or those chickens that respond negatively to the MCA/Bz injection, since they are properly the subject of another study. Our present purpose is served by the formation of a tumor within the technical demands of the germfree work.

Transmission of MCA/Bz-induced fibrosarcomas by tissue homogenates from germfree to germfree, germfree to conventional, conventional to conventional, and germfree to germfree/conventionalized Leghorn and Bantam chickens. Since tumors can be induced in chickens by a single injection of MCA/Bz, the question of their transmission is raised. Such tumor induction was first attempted with tumor cell homogenates, then with tumor homogenates repeatedly frozen and thawed, and finally by fractions of cells obtained by centrifugation and filtration.

The results of injecting tumor cell homogenates (TABLE 4) indicate that MCA/Bz-induced tumors could be transmitted from germfree to germfree, germfree to conventional, and germfree to germfree/conventionalized chickens. These tumors could not be transmitted from conventional to conventional chickens. A tumor 1 cm. in size was taken at first appearance in time/days. Passage 1 means that the transfer was made from a well-established (15- to 30-

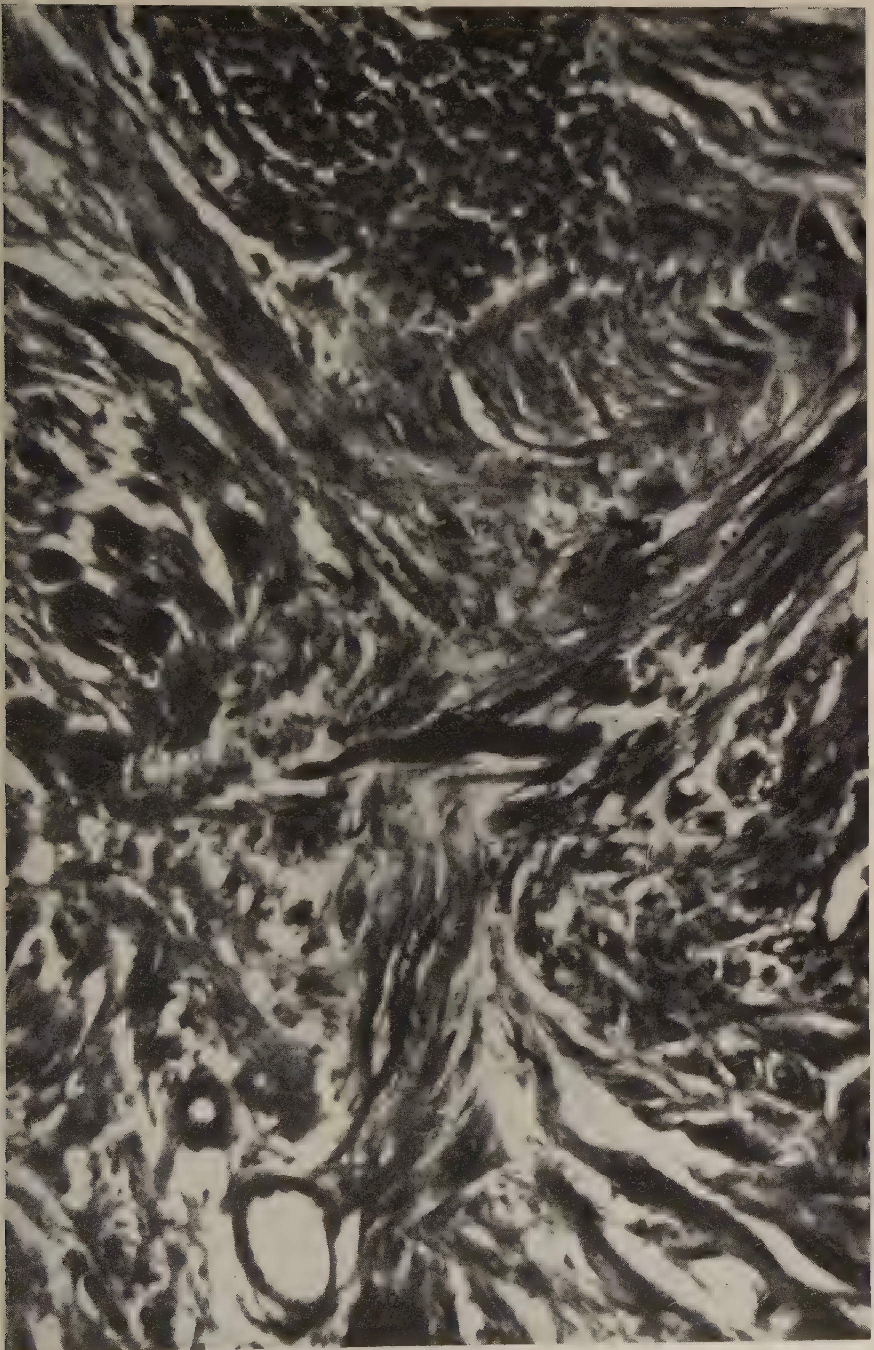

FIGURE 6. Section of MCA/Bz tumor induced in a germfree chicken. ×400.

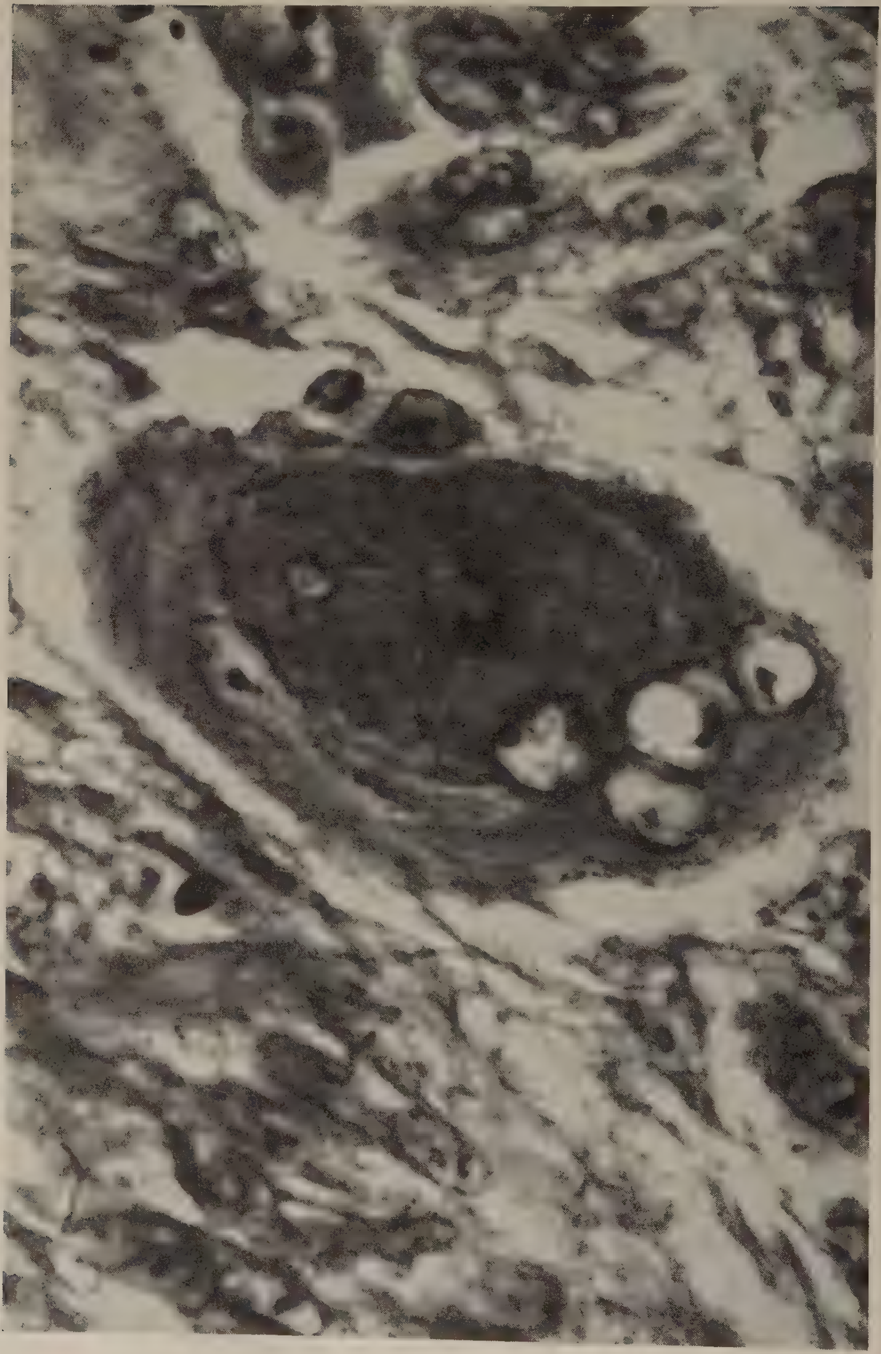

FIGURE 7. Section of MCA/Bz tumor shown in FIGURE 6. ×900.

TABLE 4

TRANSMISSION OF MCA/Bz-INDUCED TUMORS BY TISSUE HOMOGENATES AND SUBSEQUENT SERIAL PASSAGE*

Pass No.	Source of inoculum	Tumor incidence per No. used	Days postinjection to tumor appearance			Source of inoculum	Tumor incidence per No. used	Days postinjection to tumor appearance		
			Max.	Min.	Av.			Max.	Min.	Av.
	Leghorns					Bantams				
Germfree to germfree										
1	MCA, GF, L TU	16/20	60	15	22	MCA, GF, B TU	28/29	40	6	16
2	Pass. 1, GF, L TU	10/10	14	8	11	Pass. 1, B TU	5/5	10	4	5
3	Pass. 2, GF, L TU	8/8	13	8	9	Pass. 2, B TU	4/4	90	15	39
4†	Pass. 3, GF, L TU	7/7	14	2	6	Pass. 3, B TU	7/9	30	16	24
						Pass. 4, B TU	7/7	24	5	11
						Pass. 5,‡ B TU	11/11	27	14	17
Germfree to conventional										
1	MCA, GF, L TU	5/21	200	27	82	MCA, GF, B TU	9/20	60	6	32
2	Pass. 1, GF, L TU	7/11	37	13	20	Pass. 1, B TU	10/10	20	4	12
3	Pass. 2, GF, L TU	4/6	20	7	17	Pass. 2, B TU	4/4	16	4	8
Conventional to conventional										
1	MCA, GF, L TU	0/40	—	—	—	MCA, GF, B TU	0/19	—	—	—
Germfree to germfree/conventionalized										
1	MCA, GF, L TU	2/6	55	55	55	MCA, GF, B TU	4/6	120	72	96

* Key: MCA = methycholanthrene; Bz = benzene; GF = germfree; L = Leghorn; B = Bantam; TU = tumor.

† Discontinued.

‡ Current.

day) MCA/Bz-induced tumor. Transmission could be effected in either Bantam or Leghorn chickens or from one variety to another without difficulty. Whatever differences that might exist between the varieties are slight and not important to the present discussion.

Transmission from germfree to germfree chickens indicates a success of approximately 77 per cent for an average of 26 days to the first appearance of a tumor. There was little difficulty in transmitting this tumor through at least four passages. Currently we have achieved the eighth serial passage in Bantam chickens. The "take" time with each successive passage tends to decrease to a minimum of about 4 days. There is some evidence that chickens from 15 to 20 days of age are more susceptible than those 30 to 60 days of age, and the use of older chickens contributes to the take reported.

Attempts to transmit MCA/Bz primary germfree tumors to conventional chickens were successful, and the transmission could be continued through successive serial passages in conventional chickens. There is about 40 per cent take from the primary tumor, with an average of 54 days to the first appearance of the tumor.

The passage of primary MCA/Bz tumors in conventional chickens to other conventional chickens was uniformly negative. The conventional chickens were maintained on diet BR, which was not fortified with antibiotics. The results are the same for unfortified diets L-291 and L-289.

Tumors produced in germfree chickens that were later conventionalized and then passed to conventional chickens grew with an average take of 50 per cent, 16 days elapsing before the first appearance of the tumor. The experience of contamination does not prevent tumor formation or further passage of tumors.

All attempts to pass conventionally formed tumors to germfree chickens ended in contamination.

The primary MCA/Bz tumor was a fibrosarcoma. There is little change in histology of these tumors with passage.

Transmission of primary MCA/Bz-induced tumors in conventional chickens to conventional chickens on diets containing antibiotics. In one period of these experiments an MCA/Bz tumor induced in a conventional chicken was transmitted to another conventional bird. Since this was contrary to prior experience, the details of the experiment were examined. It was found that the diet used had been fortified by the manufacturer with purified extractives from *Streptomyces* fermentations and dried *Streptomyces* meals, whereas the ration that had been used was not fortified. It had been reported by Gordon *et al.*[14] that keeping conventional chickens from time of hatching on a diet containing procaine penicillin at a level of 50 mg./kg. diet caused them to develop morphologic characteristics qualitatively similar to those seen in germfree chickens. These changes consisted in lower weight and lymphoid tissue content of those organs normally in close association with an abundant microbial flora, such as the intestinal tract. Comparable antibiotic-treated germfree Leghorns failed to show any differences from the germfree norm. In short, antibiotic-fed conventional chickens assumed the characteristics of germfree birds.

Since conventional-to-conventional tumor passage had occurred with antibiotic-fed chickens, and because of the observations of Gordon *et al.*,[14] a series of experiments was indicated. The prime purpose was not to investigate the mechanisms and reasons behind the findings, but to obtain chickens that might be used for testing purposes, that is, to determine tumor activity outside the germfree system. This would permit the use of more chickens and relieve the pressure for testing on the small number of germfree chickens available.

TABLE 5

TRANSMISSION OF MCA/BZ-INDUCED TUMORS BY TISSUE HOMOGENATES AND SUBSEQUENT PASSAGE FROM CONVENTIONAL TO CONVENTIONAL ANTIBIOTIC-FED LEGHORN CHICKENS*

Pass No.	Source of inoculum	Diet	Amt. mg./kg. antibiotic diet	No. with tumor per No. used	Days postinjection to tumor appearance			Remarks
					Max.	Min.	Av.	
1	MCA, CN, L TU	BR	S 50	5/29	150	30	70	
1	MCA, CN, L TU	289	T 50	0/6	—	—	—	
1	MCA, CN, L TU	289	S 50	5/8	60	23	40	
1	MCA, CN, L TU	289	BI 50	2/9	45	23	35	
1	MCA, CN, L TU	289	P 50	18/39	66	30	39	
1	MCA, CN, L TU	289	P 50	0/14	—	—	—	
Passage from antibiotic to no antibiotic to antibiotic								
1	MCA, CN, L TU	289	P 50	2/3	28	21	24	
2	Pass. 2, CN, BI	289	None	2/3	17	6	11	
3	Pass. 3, None	289	P 50	3/3	30	6	18	
2	Pass. 2, P TU	289	T 50	3/3	30	18	22	
3	Pass. 3, P TU	289	P 50	6/6	19	19	19	
4	Pass. 4, P TU	289	P 50	6/6	25	7	12	
5	Pass. 5, P TU	289	P 50	6/6	12	12	12	Discontinued
Passage from antibiotic to no antibiotic								
1	MCA, CN, L TU	289	P 50	1/6	40	40	40	
2	Pass. 1, BI, L TU	289	None	6/7	30	7	11	
3	Pass. 2, None	289	None	8/8	20	7	11	
4	Pass. 3, None	289	None	6/6	20	7	9	
5	Pass. 4, None	289	None	6/6	11	7	11	Discontinued
Passage from P antibiotic to 150 mg./kg. diet								
1	Pass. 2, BI, L TU	289	P 150	0/12	—	—	—	
1	Pass. 2, BI, L TU	289	P 50	6/6	17	6	11	

* Key: P = procaine penicillin, S = streptomycin, T = Terramycin, BI = Bicillin, CN = conventional; otherwise as in TABLE 4.

Accordingly, experiments were attempted using sterilized diets L-289 and L-289/AF to which Terramycin, streptomycin, Bicillin, and procaine penicillin vere added at a level of 50 mg./kg. diet. This was the level used to promote growth in conventional chickens that had been examined by Gordon relative o the morphologic changes mentioned earlier. The results of these experinents (TABLE 5) show that, whereas tumor transfer was not affected from ionantibiotic-fed conventional to conventional chickens or to conventional hickens fed Terramycin, it was possible to transmit these tumors if the chickens vere first kept for 20 to 30 days after hatching on diets containing streptonycin, Bicillin, or procaine penicillin. There is indication that the effect is iot so pronounced or disappears at 150 mg./kg. levels of these antibiotics, which lso agrees with the findings of Gordon *et al.*[14] relative to morphologic changes.

Once a primary conventional MCA/Bz-induced tumor is transmitted to an antibiotic-fed chicken, it can be further passed in conventional chickens with or without antibiotics added to the diet. At least five passages can be carried out, which is as long as we have tried to transmit the tumor. The tumor is not altered in structure.

Transmission of MCA/Bz-induced tumors repeatedly frozen and thawed as homogenates from germfree to germfree, germfree to conventional, and germfree to germfree/conventionalized chickens. In accordance with our plan of investigation, a technique was sought that might damage the tumor tissue cell, but not the particulates, so that the cell would not multiply as a cell. The method used must be adaptable to the peculiar demands of the germfree technique; that is, it must be carried out in a germfree system. An investigation of the different possibilities indicated that for the preliminary work the most convenient method would be repeated freezing and thawing. It is fully realized, from discussions with various authorities and from the literature, that this is a controversial subject, with considerable disagreement as to whether repeated freezing and thawing will render all tissue cells in a homogenate inactive as cells.

Tumor tissue homogenate prepared as indicated previously, 30% v/v in saline, was frozen for 30 min. in a bath of ethylene glycol monomethyl ether (methyl Cellosolve)* and solid CO_2. The sealed tubes containing 10 ml. of the final homogenate were completely thawed from a 10% v/v suspension in saline, and the freezing and thawing cycles were lengthened. This produced no notable change in results insofar as tumor formation or transfer is concerned.

Repeated microscopic examination of the frozen and thawed homogenates shows few reasonably intact cells; however, these examinations are from samples and do not represent the entire injectate. Freezing and thawing has also been done with the homogenates being further ground in sand, and no markedly different results have been observed.

It might be noted in passing that storage of active tissue homogenates for thirty days or longer seem to render them inactive insofar as tumor formation is concerned. The storage problem is under further investigation.

The results of these experiments are shown in TABLE 6. It was possible to transmit tumors from germfree chickens to other germfree chickens after the homogenate had been frozen for as many as six cycles, and similar transfers could be made from germfree to conventional chickens. Such transfers can be continued through serial passages. In attempts to transmit tumors from germfree to germfree/conventionalized chickens, six cycles of freezing and thawing do not prevent the transfer from taking place. It might be pointed out further that there is no change in the kind of tumor formed or in the structure when it is compared to nonfrozen homogenates. Moreover, there seems to be little difference in take time or in the percentage take of the tumor.

It is not known how many times tumor homogenates can be frozen and thawed but, in one instance, this was done for 12 cycles with successful tumor transmission. However, there must be some practical limit, and accordingly 3 cycles were selected, since this takes approximately 6 hours to perform. The

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* Product of Union Carbide & Carbon Corp., New York, N. Y.

TABLE 6

TRANSMISSION OF TUMOR HOMOGENATES REPEATEDLY FROZEN AND THAWED

Pass. No.	Source of inoculum*	Number of freeze thaw cycles	No. with tumor per No. used	Days postinjection to tumor appearance			Remarks
				Max.	Min.	Av.	
	Germfree to germfree						
2	Pass. 1, GF, L TU	3	15/15	26	20	22	
2	Pass. 1, GF, L TU	0	6/6	20	20	22	
2	Pass. 1, GF, L TU	6	4/5	24	14	14	
2	Pass. 1, GF, L TU	1	0/5	—	—	—	Stored 22 days
	Germfree to conventional						
2	Pass. 1, GF, L TU	3	18/26	20	10	12	
3	Pass. 2, GF, L TU	0	10/10	11	9	10	
	Germfree to germfree/conventionalized						
2	Pass. 1, GF, L TU	3	9/11	22	13	15	
2	Pass. 1, GF, L TU	6	4/5	26	13	16	
2	Pass. 1, GF, L TU	0	3/3	22	20	21	

* Key: GF = germfree ; L = Leghorn; TU = tumor.

important point is that repeated freezing and thawing do not greatly affect transmission of tumors under the conditions of these experiments.

Transmission by filtrates of tumor tissue homogenates from germfree to germfree, ermfree to conventional, conventional to conventional, germfree to germfree/conventionalized, and conventional to germfree/conventionalized chickens. In the earch for possible subcellular particulates that will cause tumors in chickens, he technique used must be adaptable to the demands of the germfree method. As already demonstrated, repeated freezing and thawing of tumor tissue homogenates will not affect the production of tumors when injected into chickens. While freezing and thawing may not destroy the activity of tumor cells, it hould be noted also that the take time or the percentage take of such material s not greatly affected as compared to nonfrozen homogenates, nor is the structure of the formed tumor altered from that seen in those formed by unfrozen issue homogenates.

In order to study the possibility of subcellular particulates that might be ctive in tumor formation, the most usable techniques for our purpose were entrifugation and filtration. Filtration can be carried out within the confines f a germfree system but we have not yet been able to introduce a high-speed entrifuge. Attempts to remove germfree preparations from the germfree ystem for centrifugation have been only partially successful because of contamination resulting when the preparations are returned. It now seems possible to mount a high-speed Servall centrifuge inside the germfree isolator in uch the same manner as has been done with the Servall Omnimixer. However, the preliminary work reported was done using filtration which, of course, will permit a selection of particle sizes, but does not concentrate them. Consequently, the preparations used probably contained a low level of particles in the fractions studied.

For the work reported the homogenate was prepared as already described (10% v/v saline) filtered through a coarse-fritted filter (40 to 60 μ mps) and finished through a UF filter (0.9 to 1.4 μ mps). Occasionally the coarse filtrate alone was used. In a few experiments filtration was preceded or followed by freezing and thawing. The filtrates were given in a single 0.25-ml. injection into the right breast muscle of the chicken.

The results of the filtration experiments are shown in TABLE 7. In no instance when using UF filtrates was tumor formation observed. A disease, tentatively called wasting disease for want of a better term, was seen for the first time. This disease is characterized by extreme wasting of both breast muscles (FIGURE 8). The animal continues to eat well until it becomes moribund; on all animals autopsied before death, the crop was full and the intestinal tract contents normal. The muscle tissue is reduced 70 to 80 per cent from normal. Liver and lungs are normal. The gall bladder is somewhat enlarged

TABLE 7
PASSAGE OF FILTRATES FROM MCA/BZ TUMOR HOMOGENATES*

Source of filtrates	Filtration sequence	No. with tumor per No. used	No. with wasting per No. used	Approximate days to wasting
		Germfree to germfree		
Pass. 1, GF, L TU	CF-UFF	0/11	8/11	44
Pass. 1, GF, L TU	CF	16/16	0/16	—
		Germfree to conventional		
Pass. 2, GF, L TU	CF-UFF	0/27	5/27	60
		Conventional to conventional		
Pass. 1, CN, L TU	CF-UFF	0/28	0/28	—
		Germfree to germfree/conventionalized		
Pass. 1, GF, L TU	CF-UFF	0/23	3/23	72
		Conventional to germfree/conventionalized		
Pass. 1, CN, L TU	CF-UFF	0/23	5/23	120
		Conventional antibiotic to conventional antibiotic		
Pass. 1, CN, AF	CF-UFF	0/21	7/21	—

[*] Key: CF = coarse-fritted, UFF = ultrafine-fritted, CN = conventional, TU = tumor, L = leghorn, GF = germfree, AF = antibiotic-fed.

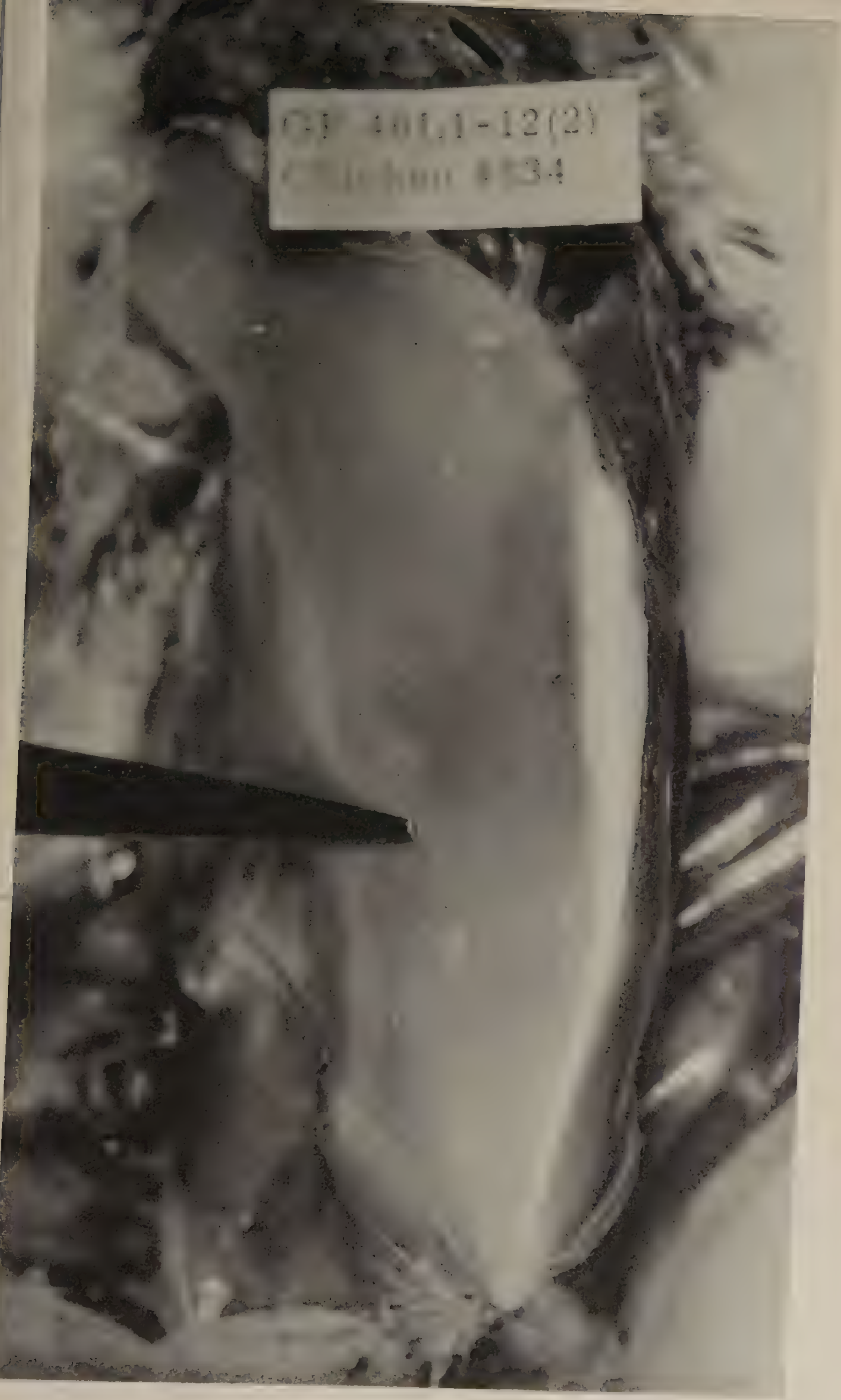

FIGURE 8. Effect of wasting disease induced by injection of UF filtrate from tumors of ermfree birds into the breast muscle of a germfree chicken.

and contains normal bile. The spleen is less than 50 per cent normal. Peyer's patches are macroscopically absent. The lymphatic system is disrupted and reduced in size. The thymus is absent. The trident is unusually small. The testicles are normal, as are the kidneys. The adrenals are large. The brain, spinal cord, and meninges are normal. The bursa is flabby, and the proventriculus enlarged. No changes are visible at the site of injection. The nerves appear normal. The marrow appears yellowish, fatty, and aplastic. The thyroid appears normal.

The most conspicuous changes are in the muscles and in the lymphatic system. There are no conspicuous lesions, and no tumors have been observed on careful examination.

The birds eventually succumb to this condition. We have seen no recovery once the disease has been observed, even with efforts to supplement the diet by proteins or vitamins. Neither have we seen this disease in uninoculated chickens, so that it does not seem to be transmitted even in the close confines of the germfree isolator. Controls injected with saline have not shown the changes described. The disease does not seem to be associated with the diets used, all of which are quite adequate for normal growth, as is shown by the controls and in the development of the chickens up to the time they are inoculated and for several weeks following inoculation. Inoculated chickens not showing the disease appear normal and grow normally.

Coarse filtrates of germfree tumor tissue passed through either C-fritted glass or C-Seitz pads will cause tumors, and these can be passed further in germfree chickens by coarse filtrates. In only a few instances have we seen wasting and tumor in the same chicken.

The UF filtrates of tumor homogenate regularly produce wasting disease when passed from one germfree chicken to another. Passage through UF filtrates from germfree to conventional chickens also causes wasting, but no tumors. In passages from conventional to conventional chickens, neither wasting nor tumors was observed. Passages from germfree to germfree/conventionalized chickens produced wasting in a few instances, but no tumors. One of the cases of wasting in the latter category occurred in chickens with a history of 129 days' germfree life postinjection. Passages from a group of conventional to germfree/conventionalized chickens yielded no tumors, but wasting was seen in a number of these birds. Passages of the tumor UF filtrate from conventional antibiotic-fed to conventional antibiotic-fed chickens showed wasting, but no tumors.

Mention should be made that the wasting disease has been transmitted by injecting serum from a wasted germfree Leghorn as a single 0.25-ml. injection into the right breast muscle of germfree Leghorns. The take time of this serum passage was about twice as long as with the filtrates from tumors. Wasting has also been transmitted with the UF filtrate from a homogenate of MCA/Bz germfree Leghorn tumor to antibiotic-fed conventional Leghorns. Brain tissue homogenate from a wasted germfree Leghorn, when injected as a homogenate or a UF filtrate into conventional antibiotic-fed chickens, yielded negative results from the homogenate and wasting from the filtrate. Control birds injected with similar preparations have been negative for both tumors and wasting. Thus, the wasting disease can be passed from chicken to chicken through UF filtrates from brain homogenates or sera of wasted chickens.

From these preliminary results it appears that a subcellular factor associated with tumor cells that will pass through UF filters will cause a wasting disease, while coarse filtrates from tumor homogenates will cause tumors. These results are suggestive and, while they are not definitive, they warrant further attempts to concentrate the fractions by ultracentrifugation and to study the fractions in the electron microscope. Preliminary results of such studies are reported elsewhere in this monograph by Taylor *et al.*

Summary

Germfree. Tumors will develop following an injection of Rous sarcoma (bacteria-free) UF filtrate. Spontaneous tumors have not been observed within the limits of the experimental period. A tumor can be caused by a single injection of MCA/Bz into the right breast muscle. Such tumors can be passed to other germfree chickens by injecting tissue cell homogenate and the passage continued. Repeated freezing (−70° C.) and thawing (25° C.) for 6 cycles does not prevent the tumor homogenates from causing tumors, nor does it alter the take time or kind of tumor when injected into other chickens. Coarse filtrates (40 to 60 μ mps) of germfree tumor homogenate will cause tumors. Ultrafine filtrates (0.9 to 1.4 μ mps) will not cause tumors, but will cause a disease characterized by extreme muscular wasting of the breast muscle. This disease can be transmitted to other chickens through serum or from UF filtrates of brain homogenate from wasted chickens. Controls injected with tissue from nonwasted chickens are negative.

Germfree to conventional. Tumors will develop following a single injection into the right breast muscle of tumor homogenate from germfree birds into conventional chickens. These tumors can be passed as homogenates to conventional chickens and continued as passages in conventional ones. Repeated freezing and thawing of the germfree tumor homogenate will also produce tumors in conventional chickens. Ultrafine filtrates from germfree tumor homogenate will produce wasting in conventional chickens, but no tumors.

Conventional to conventional. Tumors can be caused by MCA/Bz in conventional chickens. Attempts to pass these tumors as homogenates to conventional chickens have been negative. Also negative are attempts to cause tumors from coarse filtrates or wasting from UF filtrates of conventional tumor tissue homogenate injected into conventional chickens.

Conventional antibiotic-fed chickens. While attempts to transmit tumors from conventional tumor homogenate to conventional chickens have been negative, such a transmission can be effected if the chickens are fed on antibiotics (50 mg./kg. of diet) from hatching. This transmission can be continued either in conventional antibiotic-fed chickens or conventional chickens not fed the antibiotics. The effect is lessened or lost when the level of antibiotic reaches 150 mg./kg. of diet. The wasting disease can be developed from UF filtrates of conventional antibiotic tumors in conventional antibiotic-fed chickens.

Germfree to germfree/conventionalized. Ultrafine filtrates from Rous sarcoma will cause tumors in germfree chickens but, if the chickens are conventionalized, that is, permitted to become fully contaminated by removing them to a room environment, tumors either will not develop or will be altered. This seems to depend upon the length of germfree life after injection. Tumors will develop in MCA/Bz-injected chickens in this category, but the development is not greatly influenced. The tumors that form can be transmitted to conventional chickens. Freezing and thawing germfree/conventionalized tumor homogenates does not greatly influence the tumor-producing properties. Ultrafine filtrates from germfree tumor homogenates will cause wasting in germfree/conventionalized chickens.

Conventional to germfree/conventionalized. None of the attempts to pass conventional tumor tissue to germfree chickens were successful without contamination of the germfree chickens. Ultrafine filtrates from conventional chickens to germfree/conventionalized chickens resulted in no tumors, and wasting was observed in one group.

Discussion

As previously mentioned, no work has been reported on germfree chickens and tumorigenesis. However, the literature is fairly extensive on chemically induced tumors in conventional chickens; a recent report by Peacock[16] summarizes this. In the work of Peacock and his group the experience has been that very few chemically induced conventional sarcomas have been transmitted by tissue and then through only one passage. Cellfree filtrates have yielded negative results.

The question has arisen concerning the presence of a virus that causes lymphomatosis, but exists in a carrier state in the chicken.[17] This question does not seem pertinent to the present experiments. The tumors here are fibrosarcomas formed at the site of injection in a relatively short period of time; they are not changed in structure on repeated serial passage.

It seems obvious that the problem with chickens is at a point where the effort, as far as subcellular particles are concerned, lies in characterizing and concentrating these cell fractions. This is what we are attempting to do with the collaboration of A. R. Taylor and his associates at Parke, Davis & Company, Detroit, Mich.

In attempting to reproduce the phenomenon outside the germfree system by the use of antibiotics fed to chickens, a means has been presented for checking these results, at least in part. On the basis of experience, however, we feel that the germfree animal provides a unique tool, and this is what we set out to demonstrate in this monograph.

Acknowledgments

We acknowledge with thanks the contributions in advice and materials from C. O. Prickett of the Stine Laboratory. His long interest in our work has been very helpful, especially in characterizing the tumors. The collaboration of Alton R. Taylor and his associates from the Virus Research Division, Parke, Davis & Company in characterizing the particulates from the tumors is acknowledged. Robert Sieczko has been in charge of sterilization and operations of the germfree equipment in these studies. We also appreciate the assistance of the technical staff of the Lobund Institute and the interest of H. A. Gordon of the research staff.

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FRACTIONATION STUDIES OF TUMOR TISSUES FROM GERMFREE CHICKENS

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The reduction in "take" time with the repeated passage of homogenates of the original chemically induced tumor in germfree birds, described elsewhere by Reyniers[1] suggests that, during the course of the repeated passages, an increase in the pathogenicity or in the quantity of an infectious agent in the tumors occurs. This thinking is, of course, based upon the concept that such neoplastic growths and viral infections are caused by the interaction of the host and an independent infectious agent. For the present let us ignore the possible source and the initiation of the process and examine the tumor itself. The first question that arises seems to be: "Do these tumors contain what we consider to be virus or viruslike particles?" We undertook ultracentrifugal fractionation and electron microscopic studies of the fractions for such orientation purposes, and we present some results of these studies correlated, as far as possible, with the biological work described elsewhere by Reyniers.[1]

It must be pointed out that no strictly quantitative comparative analyses have been carried out; instead, our object has been to obtain some fundamental information regarding the submicroscopic morphologic characteristics of these tumors. For the initial experiments, frozen tumor tissue was finely minced, frozen and thawed 3 times, and then homogenized in a Waring Blendor to yield a 25 to 30 per cent suspension of tissue in phosphate-buffered saline solution. The homogenate was then diluted to a 7.5 to 10 per cent suspension, after which it was angle-centrifuged at 4000 g for 15 min. to obtain an opalescent extract of the tumor tissue for further fractionation. No filtration or other preliminary clarification was used in these early experiments. Later, after learning something of the gross morphologic composition of the crude extracts, the 30 per cent blender homogenate was ground in a mortar with carborundum and then filtered through nylon with No. 503 Celite as a 10 per cent suspension. Still later a modification of the fluorocarbon emulsification technique first described by Gessler *et al.*[2] was used to eliminate lipid and extraneous protein material from the extracts prior to the centrifugal fractionation.

An ultracentrifugation sequence was used (20,000 rpm for 45 min. and 40,000 rpm for 90 min. in a Spinco No. 40 rotor) similar to the cycle used in earlier work to separate equine encephalomyelitis virus[3] from a normal or microsomal component[4] of chick embryos. It was found that there was a progressive increase in the total quantity of the particulate (microsomal) fraction sedimented at 40,000 rpm from the crude saline extracts of the tumors obtained from successive passages. All preparations for electron-microscopic examination were prepared by allowing a small drop of thirtyfold concentrated pellet solution (from the original 7.5 or 10 per cent tissue extract) to filter through a collodion membrane on 1-cu. mm. agar block;[5] unless otherwise indicated, the concentrates used to make the microscope preparations were sedimented twice at 20,000 or 40,000 rpm.

FIGURE 1 shows an electron micrograph of the microsomal (40,000-rpm) fraction obtained from normal chicken breast muscle. Only a few particles in the size range of 20 mμ are visible. Strictly speaking, this does not constitute a control for the corresponding tumor fraction concentrates to follow. However, keeping in mind the degree of concentration at which we shall compare the following micrographs of the tumor fractions, it will be observed that it would be necessary to reconcentrate this normal concentrate several hundred times n order to make it comparable in number of particles per field.

FIGURE 2 is an electron micrograph of the 40,000-rpm concentrate from an original methylcholanthrene (MCA) tumor. Compared to the normal muscle concentrate seen in FIGURE 1, there is a marked increase in the number of 20-mμ particles. Wherever the value 20 mμ is used it refers to a range of particles varying in size from 10 to 35 mμ.[2] There are also amorphous aggregates not observed in FIGURE 1.

FIGURE 3 is a micrograph of the 40,000-rpm sediment from a third-passage tumor. This neoplasm appeared approximately 10 days after inoculation of the birds with the second-passage homogenate. This phenomenon is in contrast to the average time of 50 to 75 days for the appearance of the original MCA tumor, the corresponding concentrate of which is shown in FIGURE 2. There can be little doubt that there is a great deal more of the approximately 20-mμ material in this third-passage concentrate than in the original tumor concentrate. FIGURE 4 is a micrograph of another third-passage tumor concentrate.

This fraction obtained from germfree chicken tumors will produce a "wasting lisease" in germfree and antibiotic-fed conventional birds. It is of especial nterest that Reyniers and Sacksteder (elsewhere in this monograph) were unable to produce tumors with ultrafine filtrates of active tumor homogenates. However, their use of such filtrates did produce the wasting disease phenomenon in germfree and antibiotic-fed birds, but not in nonantibiotic-fed conventional birds. Furthermore, these investigators found that only coarse filtrates f the tumor homogenates will cause the growth of tumors. This latter finding, long with the fact that the ultrafine filtrates that would contain predominantly he microsomal fraction seen in FIGURES 2, 3, and 4, suggests that the tumor-inducing agent itself is retained by the filters in the coarser aggregates, or that here is a curious relation between the microsomal fraction and the whole crude issue homogenate. Accordingly, when the 20,000-rpm fractions were examined n the same manner as described above, a possible and very suggestive reason or the removal of the tumor-inducing capacity by filtration became apparent.

The twice-sedimented 20,000-rpm fractions yielded electron micrographs such s the one seen in FIGURE 5. It was immediately apparent why filtration, specially ultrafine filtration, removed the tumor-inducing capacity if it is associated with the larger particles seen here. What better filter-clogging material ould one expect to find? Additional ultracentrifugal fractionation was at-

FIGURE 1. Microsomal particles in the 40,000-rpm concentrate from the normal chicken breast muscle. ×60,000.

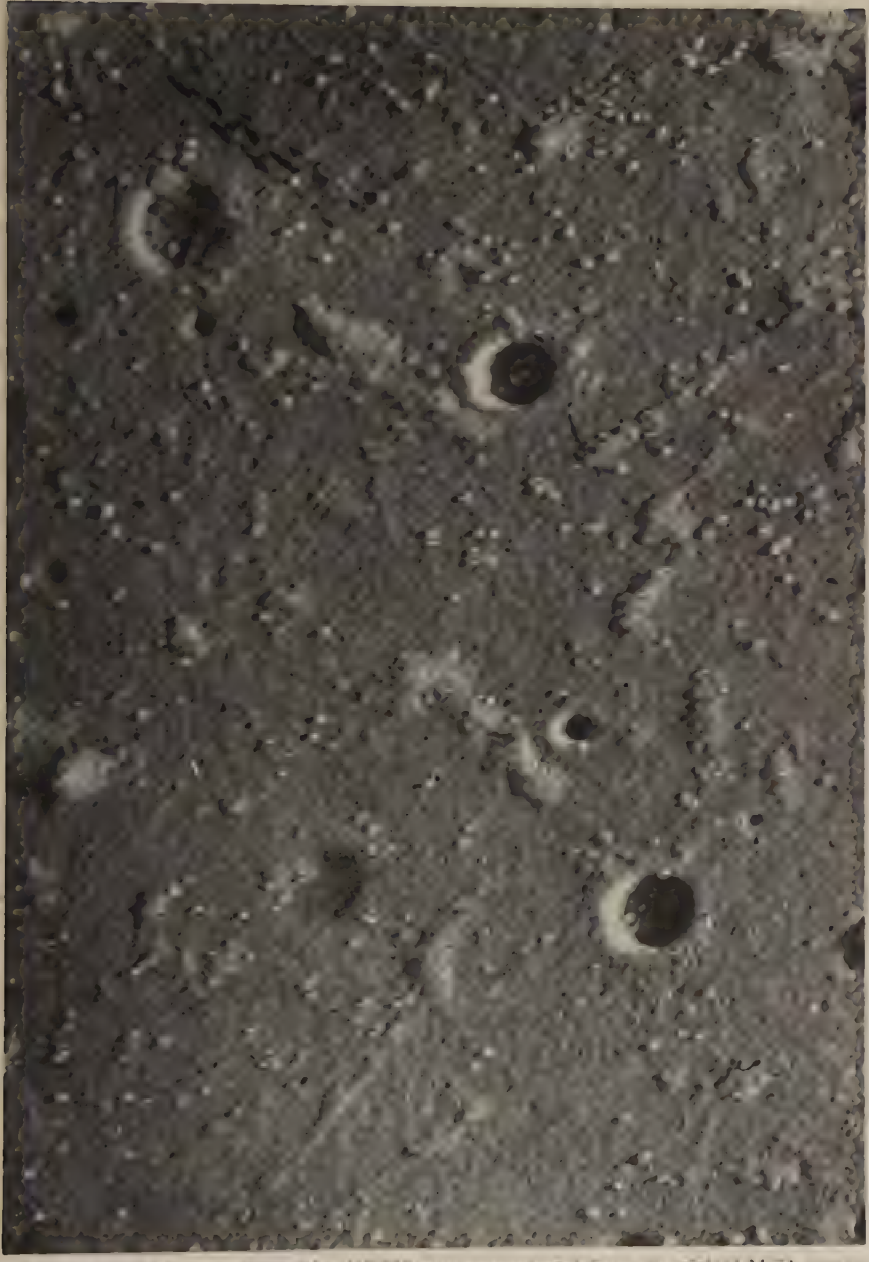

FIGURE 2. Microsomal particles (40,000 rpm concentrate) from an original MCA tumor. ×60,000.

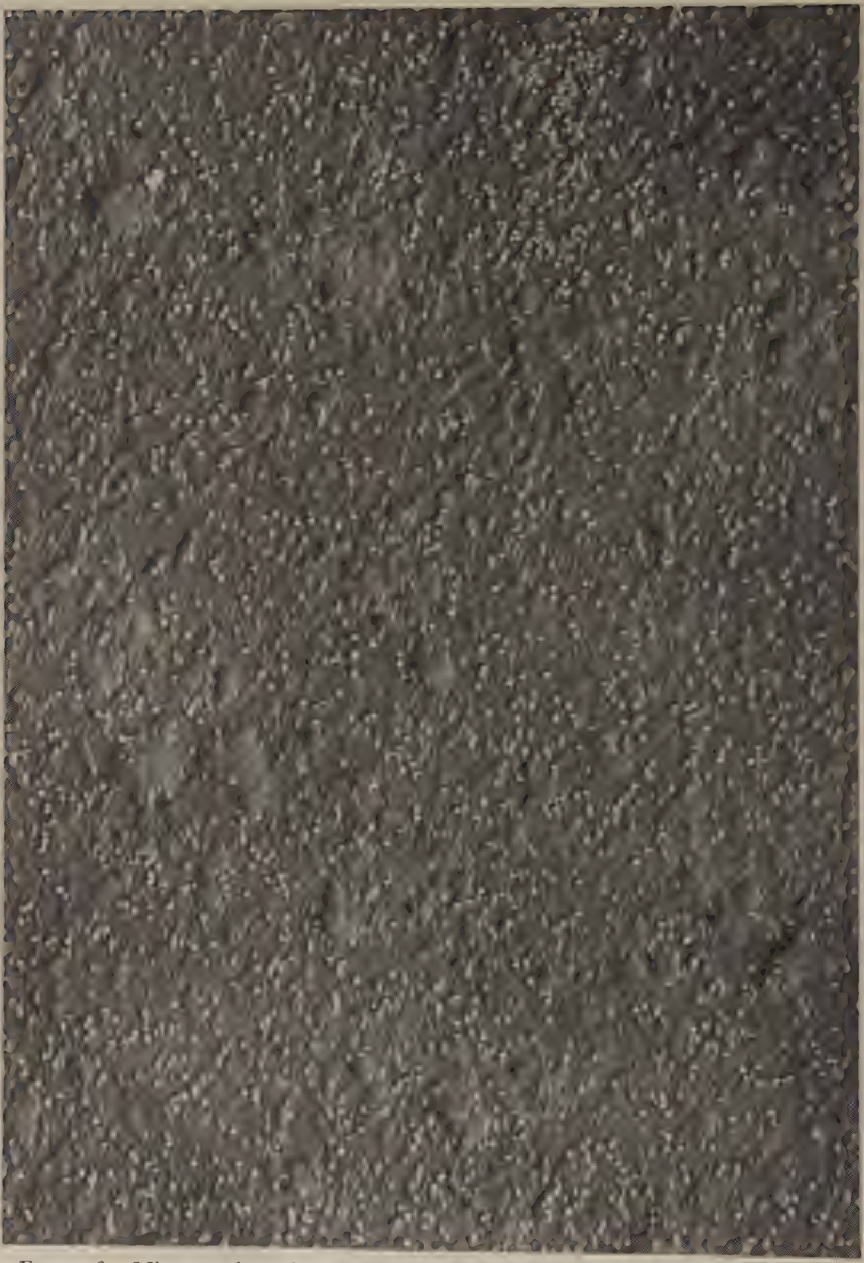

Figure 3. Microsomal particles (40,000-rpm concentrate) from a third-passage tumor. There is a marked increase in the number of particles observed in comparison to the number obtained in the comparable concentrate of the original MCA tumor shown in figure 2. ×60,000.

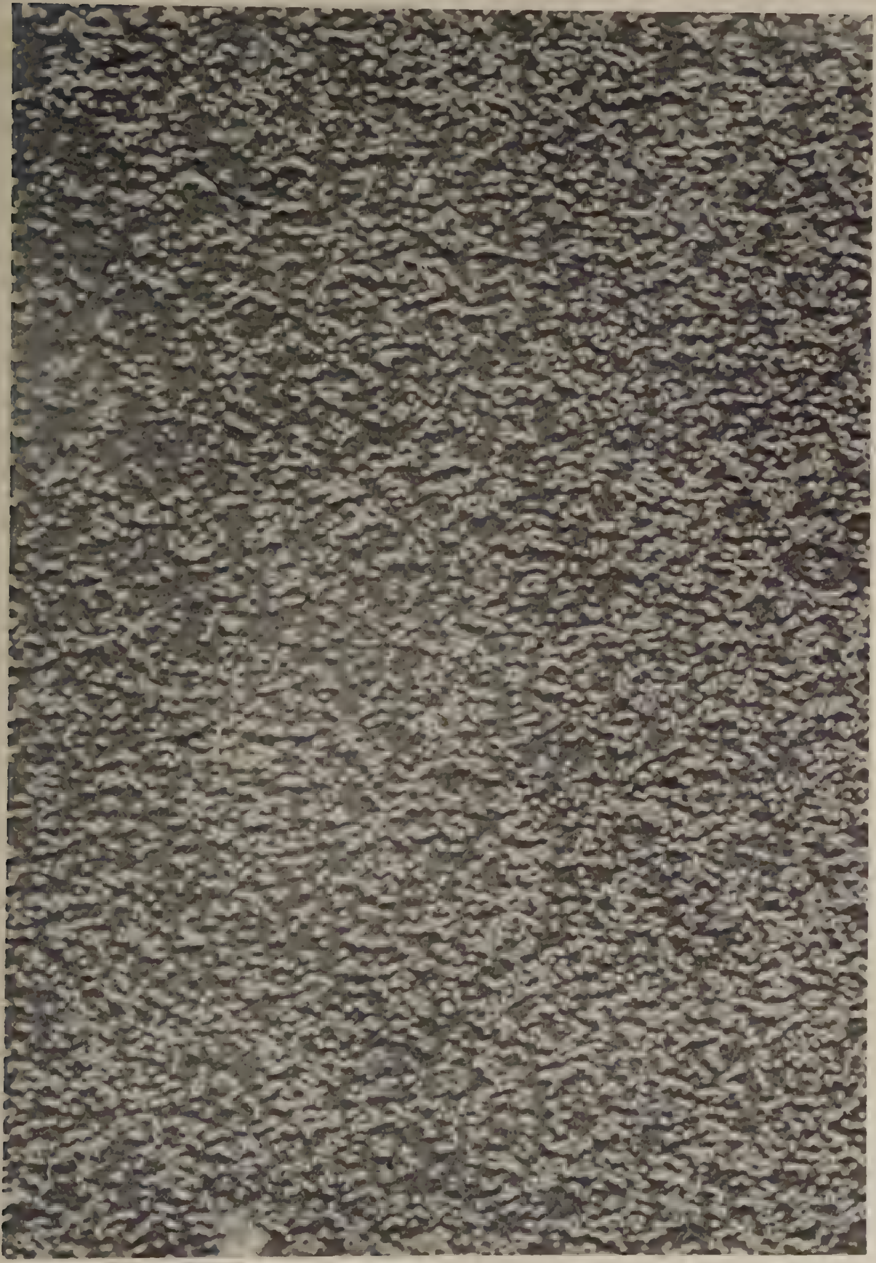

FIGURE 4. Microsomal particles (40,000-rpm concentrate) from a third-passage tumor. ×60,000.

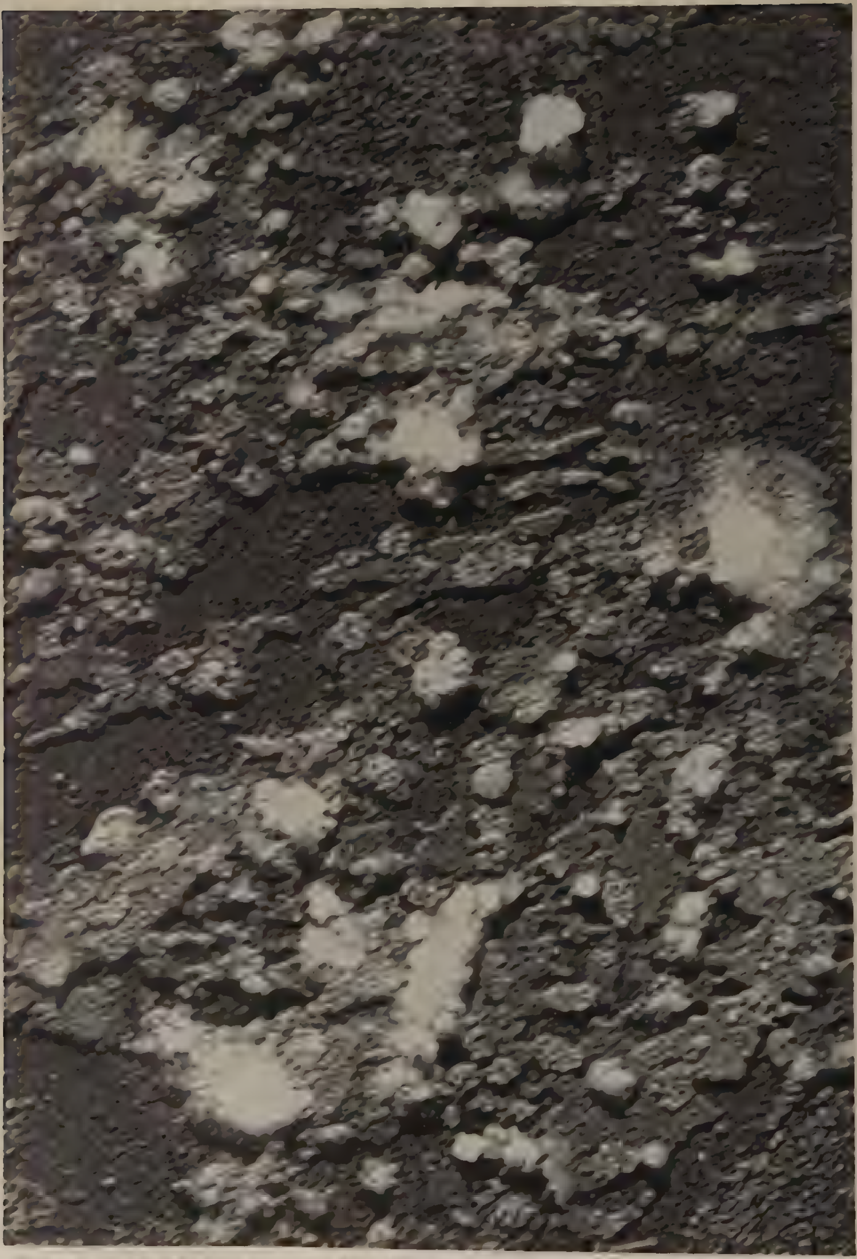

FIGURE 5. Particulates observed in twice-sedimented 20,000 rpm fractions. In addition to small numbers of microsomal particles, many larger bodies, 50 to 150 mμ in diameter, are intermeshed with a gelatinous fibroidlike material. ×60,000.

tempted by resuspending the initial 20,000-rpm pellets from the crude extract by vigorous pipetting in small volumes, sedimenting at 4000 rpm, and removing the supernatant. After this operation was repeated three times, the combined supernatants were resedimented at 20,000 rpm for 30 min. FIGURE 6 is the electron micrograph of such material. It appears that particulates of the order of 50 to 150 mμ are being released from heterogeneous material such as that shown in FIGURE 5. In addition, there are amorphous aggregates; many particles appear to be extremely flattened and have a lysed appearance. This phenomenon indicates that many of the particles are being damaged. Furthermore, the over-all concentration has been drastically reduced by this treatment.

The carborundum and nylon gauze filtration with Celite was introduced and, following 2 sedimentations at 20,000 rpm, micrographs such as the one shown in FIGURE 7 were obtained. Here we begin to see a suggestion of definitive particles in the size range of 50 to 150 mμ. Many of the larger bodies appear to be aggregates of smaller particles and there are, of course, many other particulates present. There is an indication of aggregation of 20-mμ particles and a number of the 20-mμ particles appear to line up with uniform spacing along fine fibrils, that are probably nucleic acid. This is probably a drying artefact that occurs during the specimen preparation.

The introduction of the increased homogenization by means of grinding with carborundum and the use of Celite to remove extraneous tissue debris appeared to produce much cleaner extracts that yielded concentrates containing high concentrations of definitive particles. We attempted to utilize homogenization in the presence of Genetron,* the use of which Gessler *et al.*[2] have described in connection with extraction studies on vaccinia virus and Rous sarcoma. Genetron treatment had been used with samples of germfree chicken tumor and normal germfree chicken tissue in an attempt to determine whether the tumor tissue extracts contained specific antigenic material. The rationale would appear to be that the Genetron treatment would remove nonspecific protein. The microsomal fraction appears to be capable of producing specific antisera.

Roughly minced germfree chicken tumor tissue, 30-per cent suspension in saline, was homogenized in a Waring Blendor with one-half volume of Genetron No. 226 (now designated as Genetron No. 113) for 5 min. The resulting creamy homogenate, which was centrifuged at 2000 rpm for 10 min., resulted in the separation of 3 distinct layers: (1) an upper opalescent aqueous phase, (2) an intermediate phase of coagulated protein, and (3) a lower phase of Genetron. The upper, aqueous phase was removed and used as starting material for the same ultracentrifugal sequence described above in the earlier experiments.

FIGURE 8 shows a 2-cycle 20,000-rpm concentrate from such a Genetron extract. Here one can see that there are variably sized particles in the range of 50 to 150 mμ in diameter. Nevertheless, the morphologic characteristics of these particles as observed in FIGURES 8, 9, and 10 are suggestive in spite of the variation. Some particles are only moderately flattened and appear to be

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* Genetron is the name of a series of fluorinated hydrocarbons produced by the General Chemical Division of Allied Chemical & Dye Corp., New York, N. Y.

FIGURE 6. Electron micrograph of 20,000-rpm concentrate shown in FIGURE 5 after reextraction and resedimentation; many particles appear damaged, and over-all concentration is reduced. ×60,000.

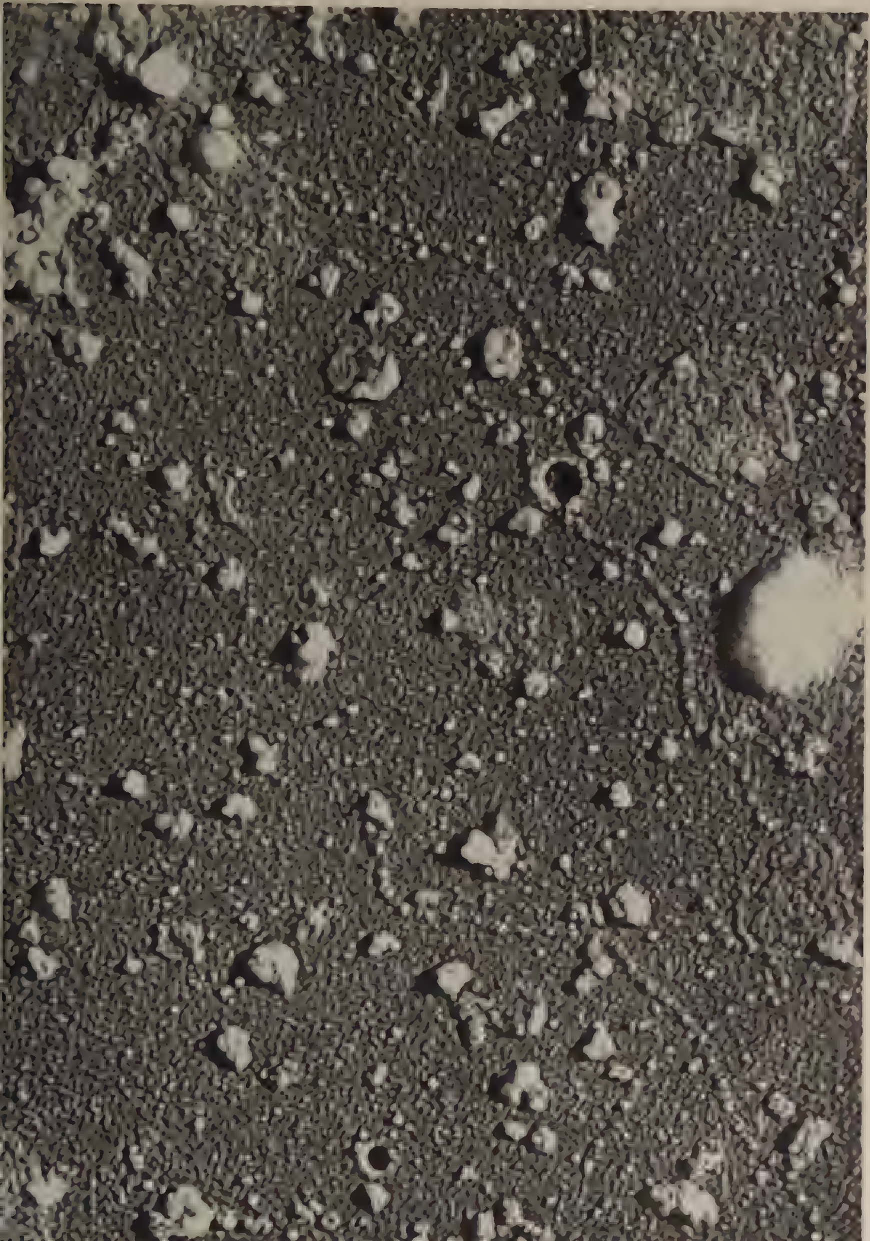

FIGURE 7. Electron micrograph of 20,000 rpm concentrate of nylon gauze filtrate of tumor xtract. Much of the gelatinous and amorphous material seen in FIGURE 6 is absent, and here appears to be more definition in the 50- to 150-mμ range. ×60,000.

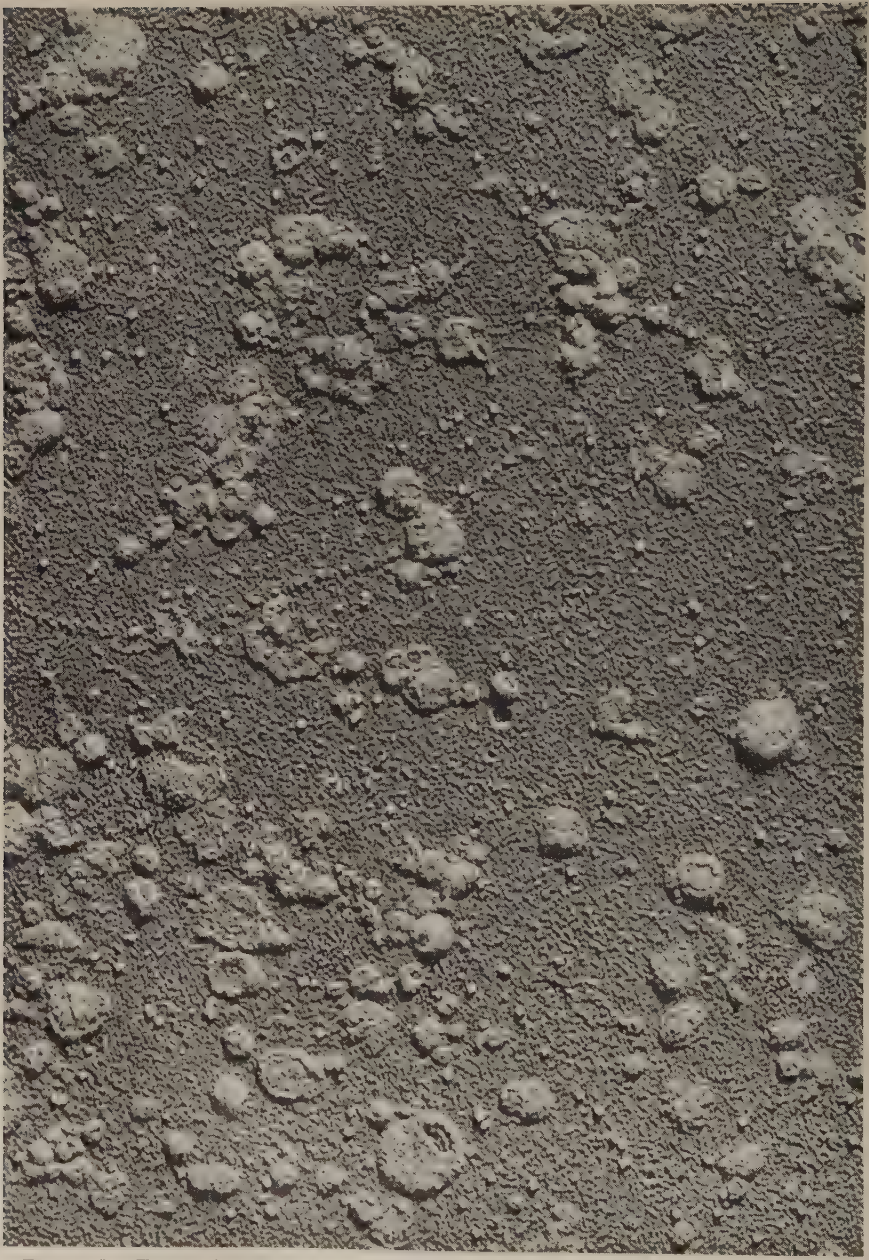

FIGURE 8. Two-cycle 20,000-rpm concentrate from a Genetron-treated tumor extract. There is considerable variation in particle size, but also a suggestion of internal structure. ×60,000.

similar to influenza virus particles. Others appear to be collapsed as though some internal material had been released.

FIGURE 9 shows a micrograph of a similar preparation, but in this case the drop of suspension apparently dried down considerably more than the preparation seen in FIGURE 8; that is, longer contact with the agar resulted in the withdrawal of more fluid from the particles. Practically all of the particles appear to be collapsed in some degree.

FIGURE 10 shows still another concentrate of the 20,000-rpm fraction. Here again there is a wide variation in size, yet much of the material appears to be morphologically distinct. There is a number of partially flattened but well-formed particles; there are others in varying degrees of collapse. In many of them there is a suggestion of internal structure. The possibility that particulates such as those seen here may represent a cellular constituent in varying stages of development or debilitation is not entirely improbable. In fact, this picture is in many ways similar to a micrograph of "purified" Rous sarcoma virus published by Sharp and Beard.[6] There are also many 20-mμ particles present and, in addition, many still smaller particles. Some of the 20-mμ particles appear to be aggregates of these smaller ones. This latter fact becomes even more interesting when the 40,000-rpm concentrates from the Genetron extractions are examined.

FIGURE 11 shows such a preparation with a large number of the 20-mμ particles. In addition, however, much larger numbers of 5- to 7-mμ particles are visible. These are present in sufficient concentration to begin to form close-packed arrays.

While we performed these fractionation studies we also examined thin sections of tumor tissue in an attempt to correlate the cell morphology with the centrifuge concentrates. To date we have had but limited success with this approach, but very suggestive particles have been seen in sections of some of the later-passage tumors. FIGURE 12 shows a thin-section micrograph of concentrations of microsomal material in the nucleus, particularly along the inner surface of the membrane. In the cytoplasm are seen groups of very suggestive viruslike particles. The size of these cellular bodies is consistent with the size range of particles seen in the 20,000-rpm concentrates.

Without further quantitative studies it is not possible to establish unequivocally a specific identification of the large particulates seen in these 20,000-rpm fractions with tumor induction. Nevertheless, the high infectivity of the crude homogenates and the fact that the size range and morphologic characteristics of these particles sedimented at 20,000 rpm (even at 10,000 rpm in more recent experiments) are consistent with the concept that the capacity for tumor induction is associated with them. Furthermore, they are morphologically similar to the particles of Rous sarcoma and another avian neoplastic disease, erythroblastic leukemia.[6] In addition, small groups of viruslike particles of similar size and morphology are seen in thin-section micrographs of the passaged germfree chicken tumor.

The marked increase in the concentration of the microsomal fraction with the progressive passage of this original chemically induced (MCA) tumor pre-

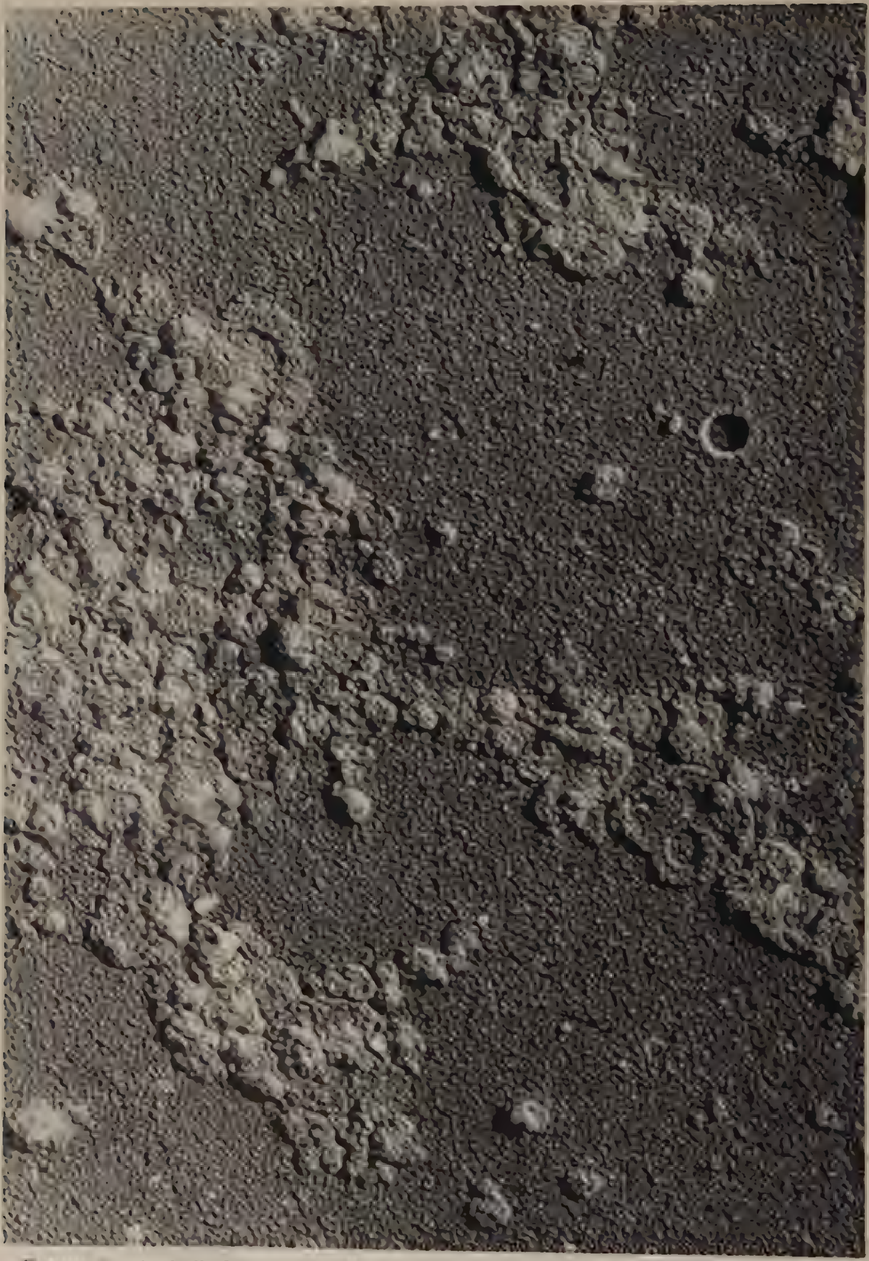

FIGURE 9. A similarly treated 20,000-rpm concentrate of a Genetron treated tumor extract showing a tendency of the collapsed particles to aggregate. ×60,000

FIGURE 10. Electron micrograph of a 20,000 rpm concentrate of Genetron treated tumor tissue where a number of partially flattened but well delineated particles in the size range of 50 to 150 mμ, as well as numerous microsomal particles, are visible. ×60,000.

Figure 11. Electron micrograph of 40,000-rpm concentrate of Geratron treated tumor extract. There are many 20-mμ particles and also large numbers of 5- to 7-mμ particles showing a tendency toward close-packed array formation. ×60,000.

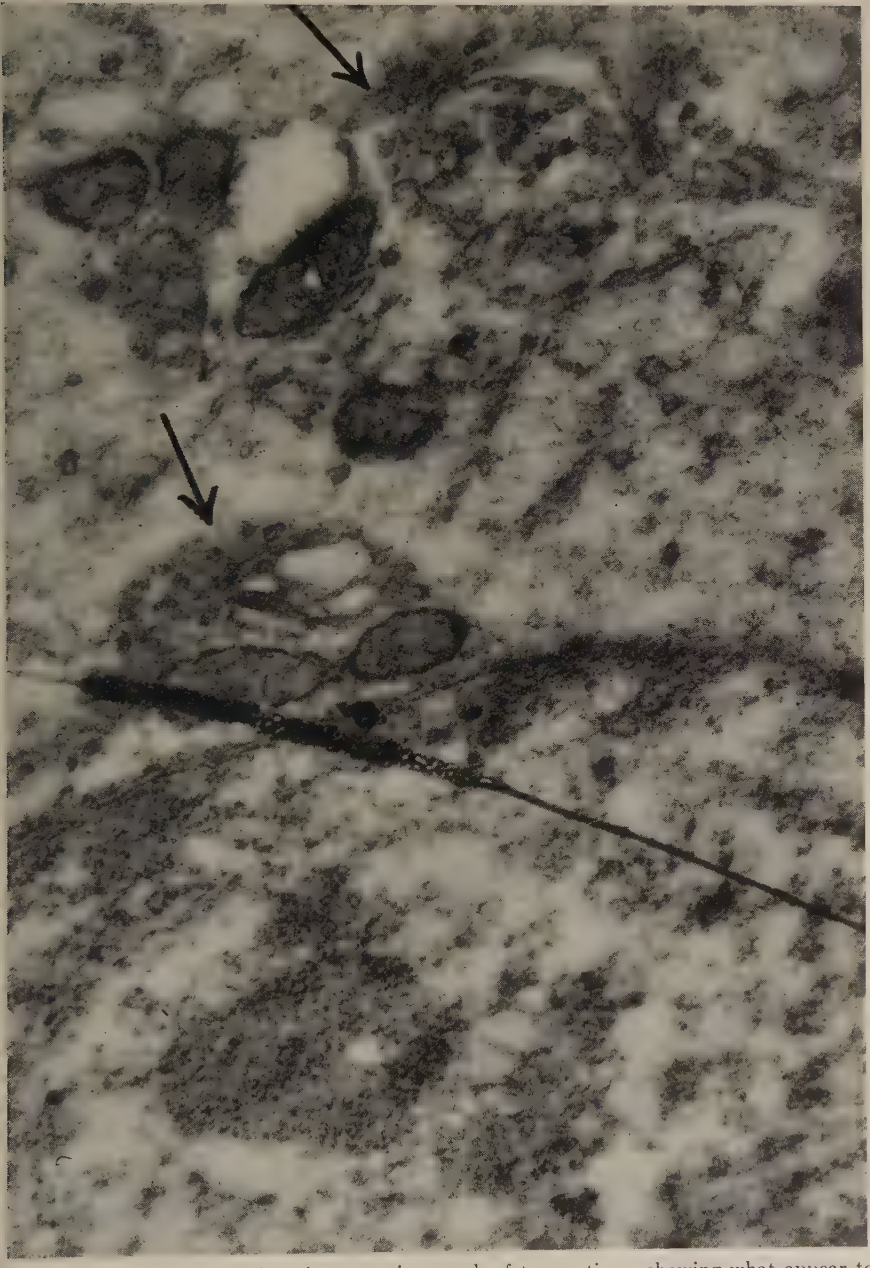

FIGURE 12. A thin-section electron micrograph of tumor tissue showing what appear to be concentrations of microsomal particles in the nucleus (lower portion), particularly along the inner membrane surface. Suggestive groups of viruslike particles are also seen in the cytoplasm. ×60,000.

sents an enigma. This is more especially the case when one considers the fact that the presence of the 20-mμ microsomal particle appears to be a characteristic component of malignant tissue culture cell lines (HeLa, HEp 2, and HEp 3). As Taylor has shown elsewhere in this monograph, we believe that this is an aspect of virology that has been somewhat neglected; perhaps we should say that there has been no indication that these submicroscopic materials are biologically active. We now have at least presumptive evidence that a "disease," namely, the wasting phenomenon observed by Reyniers,[1] may be associated with this microsomal fraction. There must be some relation between the progressively increasing concentration of this microsomal fraction seen in these experiments in germfree animals and the increasing infectivity in terms of take time for the tumor induction.

Peacock[7] finds little evidence to support viral induction of tumors, especially those induced by chemical carcinogens. No evidence for causal viruses has been found in such induced tumors, and morphologic evidence obtained by means of electron microscopy does not constitute proof for the identity of etiological and morphologic virus. We should like to reiterate and amplify the closing question of one of us (A.R.T.) elsewhere in this monograph: "Where do viruses come from originally?" Perhaps the germfree animal can help us to provide at least a partial answer to this question when we can provide certain technical improvements that will make possible the quantitative testing and evaluation of fractionation studies such as those we have just described.

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Part V. Present Status and Future Development of Germfree Life Studies

THE ECOLOGICAL APPROACH TO GERMFREE LIFE STUDIES

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Normally most animals and plants harbor a great diversity of smaller organisms, such as bacteria, fungi, protozoa, and various groups of worms and arthropods, along with viruses and other submicroscopic forms. That vertebrate animals can be freed from all or most of these has been successfully demonstrated by a number of biologists, most of whom are contributors to this monograph.

As is evident from the table of contents, germfree life studies overlap with many otherwise discrete fields of biological and medical research, while new applications are constantly being demonstrated; consequently, they represent a distinctly new method of experimentation using various ingenious techniques for rendering animals germfree and rearing them in such an environment. Today germfree animals are widely used as effective tools of research by biologists of many specialized interests. As germfree animals represent a wholly new and artificially created biotic environment, the field of animal ecology may serve as the most convenient and comprehensive frame of reference. This common point of view can then guide the various teams of specialists using germfree animals for experimental purposes.

A perfect parallel may be seen in the rejuvenation of the study of evolution (speciation) following the introduction of an ecological point of view. Thereby biologists were able to transfer the study of evolution from the traditional speculative level to an experimental one.

In characterizing the special ecological environment created through the production and maintenance of germfree animals, two kinds of problems must be resolved: first, semantic difficulties involved in expanding or adjusting existing terminologies and, second, the conceptual problems presented by the experimental animal itself.

Germfree animals are usually housed in special cages, fed and maintained in them for varying periods of time, and encouraged to reproduce their kind. The results obtained in the past three decades have disclosed no fundamental differences between germfree animals and animals not reared in this manner.

Evaluated in the light of the four kinds of relationships existing between any organism or community of organisms and the environment as stated by Raymond Pearl (see Allee *et al.*, 1949), we can now appraise the germfree environment as follows:

(1) The environment of germfree animals is even more particular and specific than is the environment of animals not living under such conditions.

(2) Germfree animals that are maintained for their entire lives in germfree condition live in a closed environment that is as continuous as is that of wild animals.

(3) The controlled environment of germfree animals exercises definite effects on its constituents and vice versa, as does the environment of wild animals; however, the germfree environment can be measured and controlled as required.

(4) Germfree life studies have demonstrated that animals can be dissociated from their normal environment, although this was long thought to be impossible. The normally indissoluble relation between animals and their environment can now be changed experimentally.

Because germfree animals are used as experimental hosts, we must also consider the new relationships that are created by the introduction of one or more microorganisms. Under these conditions new interactions between animals and microorganisms appear. Ecologists usually refer to these interactions as coactions. Molisch (1937) described many examples of coaction among plants under the term allelopathy. As defined by Clements and Shelford (1939, p. 104): "In its simplest form, each coaction comprises the reciprocal behavior of two individuals of the same or different species; the more complex coactions involve the interaction of one group or community with another." Also, bearing specifically on the situation met in germfree life studies: "In many social and symbiotic relations, the coactive organisms may exhibit more or less parity in behavior, or at least in values received."

To place the kinds of coactions met in germfree animals in their proper perspective it is best to consider them under the heading of symbiosis *sensu latissimo*, as originally proposed by DeBary (1879), and so used by Allee *et al.* (1949). In this sense, symbiosis includes three main types of coactions: (1) parasitism, in which one member is usually harmed (host); (2) commensalism, in which neither member is harmed nor is the benefit derived one-sided; (3) mutualism, in which both parties benefit from the interaction, as in the case of the protozoa found in herbivorous mammals.

Actually, "In a sense, animals as a whole are broadly symbiotic in their relations with the plant kingdom," whereas ". . . the reciprocal metabolic relations of the two kingdoms may be thought of as mutualistic" (Allee *et al.*, 1949).

In the case of germfree animals, we deal with a biotic environment on the individual level, even if experimental organisms are introduced. In other words, the status of germfree animals is a relative one, depending on whether they are being maintained as germfree animals or are being used as hosts for introduced organisms. When serving as hosts, germfree animals provide an intraorganismal or cellular environment for the introduced contaminant or contaminants.

Considered in the light of the complex interactions and mechanisms of transmission developed in insect symbiosis, vertebrate symbiosis appears to be rather simple and uniform (Rippel-Baldes, 1952). In his monograph on endosymbiosis of invertebrates, Buchner (1953) points out that the vertebrate intestine is sterile at the time of birth, but that symbionts (contaminants) enter at birth or with food, that is, from the normal environment of the animal. Buchner compares the importance of research on symbiosis with that on infectious bacteria and phagocytosis, and refers to this entire field of research as symbiontology.

A quantitative basis for characterization of coactions existing among microorganisms has been proposed by Burkholder (1952). Adopting the concepts of cooperation and conflict as defined by the sociologist Edward Haskell, he recognizes classes of possible coactions, ranging from weak to strong organisms, by using various symbols such as 0 = rate of activity, + = acceleration, and − = deceleration. Accordingly, symbiosis *sensu stricto* would be marked as ++, and parasitism as ±.

Germfree life studies must always be carried out under controlled environmental conditions. However, these conditions differ in orientation from those provided in the phytotron for controlled plant growth or similar laboratories in that they provide essentially a controlled external environment (Went, 1958). By comparison, germfree animals function as hosts of introduced microorganisms. Therefore, it was necessary to design and produce entirely different experimental plans and instrumentation to attain these aims.

The need for a new and unified terminology for germfree life studies was effectively demonstrated by Reyniers and his colleagues (1949). In making proposals for a new terminology, we must remember that a successful terminology should be clear, simple, and sufficiently flexible to allow for future expansion.

Starting with the very special kind of environment needed for germfree life studies, a term such as microecology could be formed, as were the terms microclimate and microevolution. However, the term microecology is too general. Referring to the closed and artificially limited environment used in germfree studies, a better term for this kind of environment would be stenotope, whereas the whole field of research based on it could be called stenobiology (from *stenos* —narrow). Another possible term would be cleistobiology or cleistotope, constructed as was cleistogamy (fertilization in closed flowers).

Appraising the germfree animal itself, we can safely state that it is left completely to its own resources. It could readily be called an autobiont, or an idiobiont or, less appropriately, an amicrobiont or asymbiont (without microbes or symbionts). For a germfree animal Reyniers *et al.* have proposed the term *gnotobiote*, which could be used for the germfree animal without contaminants, whereas a gnotobiote carrying one contaminant could be called a monognotobiote.

In looking over other available terms (Jackson, 1928), we find that an amotoecious plant is a parasite confined to a single host, that an aulophyte is a *Raumparasit** (the parallel term aulozoon could be constructed), and that an autoecious parasite is one that completes its whole life history in a single host. The best available term seems to be bion, as it refers to an organism that is morphologically and physiologically independent, a condition satisfactorily met by germfree animals. As "bionomics" is already in use, "biontology" is here proposed for the entire field of research dealing with all germfree forms, whether plant or animal, and their combinations as coactive systems.

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* A plant living within the cavity of another for shelter only.

As the use of embryological formulas was recommended in the field of plant taxonomy (Just, 1946), I should like to recommend for practical usage a system of biontological formulas representing a code or system of shorthand characterizations giving all essential details relative to a particular experimental animal, such as origin, operation, food, and length of life in germfree environment. If carefully worked out, this code would tell all essentials and thus facilitate reference to particular animals and situations. If desired, a germfree animal could be called a holobiont in contradistinction to one infected with a single contaminant (monobiont) or one with two contaminants (dibiont). Otherwise no single term can ever be simple enough for ordinary usage, as it would certainly be more than a sesquipedalian word. Experimental animals not living in germfree environments are best called "conventional."

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PANEL DISCUSSION

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WILLARD H. WRIGHT: In the preceding pages we have presented an array of international talent in the field of germfree life. These individuals represent nearly all of the investigators who have made contributions to this subject. All of the most recent developments have been presented. One must be impressed with the progress that has been made, and it seems that we now stand on the threshhold of a new era in this type of research, as expressed eloquently by Gösta Glimstedt.

In discussing with Reyniers the direction that this panel discussion should take, we agreed in essence that the theme should be that of the future. In other words, what are the potentials of this relatively new and unique tool, the germfree animal, in biological and medical research? With this theme in mind, the panel membership has been selected from leaders in different fields. For the most part these individuals have not been engaged in germfree-animal research. For this reason we feel that they will contribute a fresh, unbiased, and unrestrained point of view.

HENRY KUNKEL (*Rockefeller Institute, New York, N. Y.*): I shall limit my remarks to a few of the aspects of immunology with which I have had experience.

The observation by Wostmann earlier in this monograph on the low gamma-globulin levels found in germfree animals is really an exciting development and, I think, it demonstrates conclusively the important role of microorganisms in the antigenic stimulation of the host. It raises the fundamental question, "What is a normal gamma-globulin level in an animal?" Is it simply the chance result of exposure to antigens? Do hypo- and hypergamma-globulin levels represent simply variations from the mean? These observations coincide to some extent with others in different fields where elevation and depression of the gamma-globulin have been noticed and have puzzled clinicians for a number of years. This phenomenon may simply represent variations in exposure to the antigens responsible.

It would be of considerable interest to extend these observations to other classes of globulins and antibodies. For example, the antibodies of high molecular weight are arousing considerable interest at present. The isoagglutinins fall into this class and, recently, the demonstration that thyroid autoantibodies are high-molecular-weight in type aroused much comment. Many of the agglutinins that are being tested in germfree animals are of this type. It probably should be realized that these are not typical antibodies and that their cellular source is not known. There is considerable doubt as to whether the plasma cell gives rise to this type of antibody or gamma-globulin.

Microorganisms are particularly suspect in the production of this class of proteins. Such proteins can be detected by ultracentrifugation and, more recently, it has been possible to detect this kind of antibody or gamma-globulin by immunological methods. The antigenic constitution of this class is different from the ordinary low-molecular-weight class of protein.

There are other types of proteins, other classes of antibodies and gamma-globulins, as well as mixed proteins, some of which have faster motility, and some of which have particular affinity for cells. These are attracting considerable interest at present and are worthy of investigation in germfree animals. The recent demonstrations made by modifying the immunoelectrophoretic technique have provided considerable evidence regarding still other proteins of this type. All of the techniques that have been so elegantly brought out by workers in the field of agammaglobulinemia by Good, Gitlin, and their associates might well serve as models for the investigation of the germfree animal. The method of immunoelectrophoresis itself certainly could be investigated in this respect. The serum of a germfree animal could well be utilized in those studies where the complex antiserum being utilized is absorbed with the serum of an agammaglobulinemic host of this kind. This leaves just the antibodies to the deficient proteins, so that we now have an antiserum against only the proteins that are absent; accordingly, these can be detected by reacting them with normal serum. This method is really quite ingenious. It might well demonstrate other components in the serum spectrum that may be absent in the germfree animal.

The standard methods of electrophoresis are quite unsatisfactory. Charles Wuhrman illustrated this quite well when he drew an analogy between a serum electrophoretic pattern and a mountain range where the little crags at the surface represented the peaks in the electrophoretic pattern and where the great mass of the mountain represented other proteins. The method of Smithies for gel-electrophoresis, where the separation occurs on a basis of molecular size as well as configuration due to electric charge, is very promising and worthy of application to germfree animals. The theory enunciated regarding the inheritance of isoantibodies by a set of genes linked to the blood group antigens is certainly no longer tenable in view of the findings of Springer and other investigators using germfree animals. It appears clear that this group of proteins is derived from the stimulus of microorganisms. It would be interesting to see this study carried to its final completion. Similar mechanisms may be involved with respect to Forssman antibodies and the heterophile types; all the evidence indicates that these also are caused by the stimulus of microorganisms. These antigens are widely distributed in nature.

In the field of natural resistance to infection or the susceptibility to infection germfree animals, perhaps, will find their greatest role, at least from the immunological point of view.

Recent evidence indicates that bacterial cell wall material and endotoxins exert profound effects on susceptibility to infection from completely unrelated organisms. This raises the question as to whether products of the normal bacterial flora may not similarly contribute in some way to infection in general. There is no evidence for this at the present time, insofar as I understand the problem, but I think also that this is not easily attacked. These processes might be mediated through properdin as well as some of the components of complement. The problems involved might well require more study and more observations involving germfree animals.

There is also the problem of the natural opsonins, which have been known for so long a time but are still poorly understood. This is also pertinent to the discussion. The phagocytosis-stimulating principle has recently been isolated from human serum, and it exerts a marked effect on phagocytosis of such things as starch granules and nuclei, particularly in the case of the lupus erythematosus phenomenon where nuclei are phagocytized only in the presence of the accessory opsonin factor. It is not clear whether this is or is not related to the rather dramatic observations regarding phagocytosis stimulation by endotoxin. Perhaps the reticuloendothelial system is conditioned, at least in part, by varying amounts of exposure to bacterial products. This would seem to be another fertile field for study using germfree animals.

These are all highly specialized subjects in which this new tool, the germfree animal, might be used profitably. This also points to the future in which not only the germfree animal, but the antigen-free animal will be available. There is no question that continued close collaboration between the experts who rear germfree animals and the various specialists in different branches of immunology will be absolutely essential.

QUESTION: Would Kunkel care to explain or theorize on the reason that the albumin fraction is high in germfree animals and in agammaglobulinemia? I also wonder if the albumin fraction drops to a normal level when a germfree animal is exposed to a normal environment or, as Reyniers puts it, is "conventionalized?"

KUNKEL: This is an interesting point. If it has been definitely demonstrated that the albumin fraction is high, it might be pointed out that this is one of the few conditions that show an elevated albumin. Pathological states with elevated albumins do not exist. It is not found in the agammaglobulinemias. To the best of my knowledge the albumin is never high, so I am surprised that this is the case in germfree animals. I think that this is of the greatest interest, but I can offer no explanation. However, I believe that we need a great deal of evidence before we can assume that this is actually the case.

J. T. PARK (*Walter Reed Army Institute of Research, Washington, D. C.*): When the germfree rat is introduced into a contaminated environment we see the albumin, which is normally higher than in conventional animals, gradually come down to normal levels. I believe that the number of animals that we have studied established this phenomenon rather firmly. My own explanation for this has always been that the osmotic value of the blood must be sufficiently high to keep the water metabolism the same in the germfree and the conventional animal. If, accordingly, certain components of the serum protein drop in level, this may be due to a lack of stimulation; the albumin fraction more or less compensates for this to maintain the total chloride osmotic pressure at a certain value.

KUNKEL: I think that this is a very attractive theory It is surprising that this condition has not been observed in any human, but I think it is most interesting.

PARK: I believe that Gustafsson has observed this exact phenomenon in rats. We have observed something similar in chickens, but in these birds it is not nearly as marked. I know of no data on this for the human, however. Perhaps in man the situation in which this would be so evident is never approached.

BENGT GUSTAFSSON (*University of Lund, Lund, Sweden*): We have also seen this elevation of the serum albumin fraction in germfree rats, although it was not statistically significant. As there was a decrease of both beta- and gamma-globulins in germfree rats the elevation of the albumin fraction could be a compensatory one to keep the colloidal osmotic pressure of the blood at a normal level.

QUESTION: With reference to the presence of viruses in germfree animals, has anyone done a survey of the serum to determine whether it contains neutralizing antibody to a variety of viruses? One might think of latent viruses that could be present and that might explain or clarify the things that Reyniers has encountered.

A. R. TAYLOR (*Parke, Davis & Company, Detroit, Mich.*): Frankly, I cannot answer this question. I know, of course, a source of material from which the answers might be obtained. The laboratory of Karl Habel at the National Institutes of Health, Bethesda, Md., contains a vast array of sera that could be included in such a study. A certain number of tissue culture tests have been performed in our laboratory, but to date we have not detected anything. Of course, this is not a complete answer; it simply indicates another important area of research in which the germfree animal can be used.

WRIGHT: Since there has been some indication that certain specific globulin fractions are associated with immune reactions against certain groups of organisms, I should like to ask Kunkel if the evidence supports such a concept?

KUNKEL: I do not think so. I have never observed it, for example, with respect to the mobility of antibodies to different microbes. There is evidence indicating that some of the viral antibodies have a more rapid mobility, in general, than do bacterial antibodies, but I have never seen it so classified among bacteria. Most of the bacteria have a considerable amount of carbohydrates in their cell walls, and this would tend to place their antibodies in the high-molecular-weight class. In general, these have a somewhat faster mobility and fall into the gamma-1 and beta classes, so there may be some differentiation on that basis.

JAMES A. REYNIERS: (*Lobund Institute, University of Notre Dame, Notre Dame, Ind.*): Perhaps I can make a small contribution at this point. Several things have been mentioned that I believe should be clarified. One is the so-called infection of a germfree animal. Actually, many of these occurrences are not really infections, but merely contaminations. The question of pathogenicity is relative to the host, and the problem of relating this to natural immunity that might be found as a species characteristic of germfree animals is most difficult to evaluate in terms of a specific microorganism. Some measure might be had from the serologic response, but this again is relative. For example, *Shigella paradysenteriae* isolated from severe human infection and supposedly pathogenic for humans will grow, when administered to germfree rats and mice, to 10^9 in the intestinal tract within 18 hours. However, no evidence of gross pathology or sickness was demonstrated in an observation period exceeding one month.

On the other hand, as we have reported [1] with germfree C3H mice exposed to a conventionally contaminated environment (that is, the laboratory) in which animal pathogens might reasonably be expected to be absent or at least present in very small numbers, evidence of contamination and infection occurs as early as 30 to 60 min. and progress to a 50- to 60-per cent fatality within 48 hours. The cause of death was difficult to determine from a bacteriological point of view, but it was evident that a variety of normal laboratory bacteria were found in abundance in the diseased animals.

With the germfree rat it is generally true that exposure to a conventional laboratory environment has little effect on survival. Could this be due to a species difference? If so, it must be akin to natural immunity and an indication of the usefulness of the germfree animal in the study of this important problem.

While these remarks throw little light on the immunological picture, it should be noted that immunology divorced completely from resistance to infection becomes a problem in chemistry. When the problems of immunity and susceptibility are studied by challenging a germfree animal with a specific microorganism, there is much more to the study than the mere addition of an organism to the animal. Such matters as previous history and ecological factors involving association with other microbes must be considered, as exemplified by the studies of Phillips *et al.*[2] with *Endamoeba histolytica*. Perhaps Morris Wagner can add still further to this discussion.

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Morris Wagner (*Lobund Institute, University of Notre Dame, Notre Dame, Ind.*): The term natural immunity is often used rather loosely to describe certain types of immunity that appear to be innate. Some types of protection against infection appear to be inheritable, while others are more apparent than real and may be altered by changes in the physiology or ecology of the host. The origin and role of "natural" antibodies in immunity to disease have not been proved, but if there is anything to the theory that certain so-called "natural" antibodies in immunity to disease are not natural but are induced by contact with microorganisms, then the germfree animal may not develop a full complement of such antibodies. On this basis it may be possible that a given microorganism might be pathogenic to a germfree animal but nonpathogenic to a conventional animal that has had previous immunological experience through contacts with organisms in its environment.

As Reyniers has pointed out, we have contaminated germfree mice with *Shi-*

gella flexneri (types 2 and 3). This organism is classified as a pathogen because of its ability to infect man, but it generally will not establish itself in experimental animals exposed via the oral route. *S. flexneri*, when administered orally to germfree mice, rats, or chickens, in as little as 9 cells per dose, will establish a population of 10^9 shigellae per gram of cecal contents within 18 hours after inoculation. Such animals have been observed for 1 month without any sign of pathogenicity. It is noteworthy that the LD_{50} for *S. flexneri* (type 3) in conventional mice is 9×10^7 by the rectal route, which indicates that other organisms may participate in the lethal outcome of the inoculation.

On the other hand, it has been observed that *Bacillus subtilis* is highly pathogenic and lethal when introduced as a contaminant to germfree guinea pigs. Conventional guinea pigs are not affected by this organism, although they are in constant contact with it in their environment.

QUESTION: When germfree animals are brought into a conventional environment, what would happen if they were protected by inoculation with gamma-globulin from the parent? In other words, would this be analogous to the birth of a newborn baby without protective antibody from the mother?

REYNIERS: This has not been studied, insofar as I know. To answer your question with another question, if the animals have been born from germfree parents, as is usually the case, what protective effect would the serum from a germfree parent confer?

QUESTION: When some of the germfree animals are brought into a conventionally contaminated environment they will usually die within 24 hours. The question may be restated thus: if the germfree animals are given pooled gamma-globulin from the parent, will they be protected? Is this a physiological phenomenon independent of antibodies?

T. D. LUCKEY (*University of Missouri Medical Center, Columbia, Mo.*): When Reyniers and I first collaborated at the University of Notre Dame we discussed the infection of rats without actually performing any experiments, so I have observed this over the years. I have the impression, as Reyniers has said, that rats taken directly from a germfree environment to the conventional animal colony will die in 3 or 4 days. Consequently, I used to take such animals routinely into my office for 3 or 4 days before introducing them into the conventional colony, and I never experienced difficulties in this respect. On the other hand, the germfree chicken appears to show no effect, as far as fatal infections are concerned, when taken from a germfree environment and introduced directly to an environment containing conventional chicks. Apparently there is a species difference here.

GEORG F. SPRINGER (*Hospital of the University of Pennsylvania, Philadelphia, Pa.*): Antibodies occur in fractions other than the gamma-globulin.[1] With human blood group antibodies it is well known that anti-Lewis, anti-Kidd, anti-Kell, anti-M,[2] and other antibodies may be found mainly in the beta and alpha-2 fractions. The National Institutes of Health, Bethesda, Md., now request that we use the whole globulin fraction and not just the gamma fraction for the preparation of Coombs serum, since it has been demonstrated that otherwise some antibodies of importance in blood banking may be missed.

As far as natural immunity is concerned, I should like to say that the properdin system is not yet fully understood. Nelson[3] thinks that properdin may be an antibody against an almost universal carbohydrate group. It should be noted that germfree animals receive starch and cellulose in their diets.

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Gustave J. Dammin (*Harvard Medical School, Boston, Mass.*): I should like to comment briefly on the remarks made about *Shigella* and the experimental animal. I have been collaborating with Samuel Formal at the Walter Reed Army Institute of Research, where he has been working with germfree animals. I have had the opportunity to study sections on guinea pigs of the Hartley strain infected with *Shigella*-2 and *S. flexneri*-2a isolated from human cases in Japan. It is possible, by previous starvation of the animal, to produce ulcerative lesions of the colon only. Very recently I received some sections of guinea pigs that had been inoculated with the same strains. In the sections that I have seen so far, representing a small number of animals, there are focal ulcerative lesions in the bowel. What impressed me about these was not so much the defect in the mucosa, but the cellular reaction with neutrophilic polymorphonuclear neutrocytes and leukocytes and the number of eosinophils. There were eosinophils in the lamina propria quite distant from the mucosal defects.

Elsewhere in this monograph Newton and his collaborators have shown that in the germfree guinea pig receiving an inoculum of a nematode for which it is not a definitive host there is a reaction in the lung, characterized by the presence of many eosinophils. What is the role of the eosinophil in the germfree animal? Does it react to injury? Does it have any role in the immune reponse to the organisms mentioned? Perhaps Kunkel might wish to comment upon the role of the eosinophil in the immune reaction. Does it play a role perhaps only second in importance to that which we assign to the plasma cell?

Kunkel: This is a very interesting idea, and I believe that some investigators are beginning to implicate the eosinophil increasingly in immunological phenomena. As yet the evidence is not too strong with respect to the production of antibodies, but there is certainly some association of these phenomena with it.

Werner Braun (*Rutgers, the State University, New Brunswick, N. J.*): I shall confine my remarks to a few systems that illustrate the dependence of host-contaminant interactions upon prior experience of the host with contaminants. These obviously represent systems in which the germfree animal could be used beautifully to elucidate some of the very fundamental questions that still face us.

Many of the immune systems that require activation are quite well known, and I shall cite only some of them; in doing so, I shall state first that it does not appear too surprising that these systems require activation when it is recognized that many of the antimicrobial host factors, particularly bactericidal factors, are probably enzymatic in nature. It is well established, of course, that many enzyme systems do require activation either by exposure to the appropriate substrate or, at times, by removal of inhibitory factors.

In discussing activated antimicrobial host systems, I propose to begin by underscoring something that has been stated by Wagner; namely, that the germfree animal represents an ideal system to help us differentiate between specifically induced antimicrobial host factors and so-called natural or normal antibodies. The latter may well be *naturally induced* antibodies, that is, antibodies or antimicrobial factors that are produced in response to a widely distributed antigen. However, there also may exist truly natural antimicrobial host substances which, although they are part of the normal, innate make-up of the mammalian host, may act against bacteria because they affect sensitive sites of bacterial cells. This may be illustrated by reference to antibacterial systems in which complement participates. We know of at least one component of the complement system that can be converted into an esterase; and it is now quite clear that enzymes such as peptidases and esterases may be able to affect sensitive sites on the bacterial cell wall. For example, in our laboratory it was possible to demonstrate during the last year that the bactericidal activity of normal human serum, which is dependent on the complement system, can be abolished completely if the usually sensitive bacteria are suspended in serum containing 20 per cent sucrose.[1] Under these conditions survival occurs with beautiful protoplast-, or better: spheroplast-formation. The normal serum appears to contain enzymes capable of lysing out certain components of the bacterial cell wall. Under ordinary environmental conditions, that is, in the absence of a hypertonic environment, the bacteria will lyse, but when they are protected by a hypertonic environment, as produced by 20 per cent sucrose, there will be no lysis and there will be complete survival of the bacteria.

Normally present host factors that are capable of affecting cell-wall components of bacteria would represent truly natural antimicrobial host substances, but even they may require activation. Space does not permit discussion of the very interesting subject of complement activation in greater detail. It is most probable that complement is not active *in vivo* systemically, but requires for its activation antigen-antibody reactions. We know relatively little about the activators of complement systems that in turn serve as activators of an *in vivo* bactericide. Here, I think, germfree animals might give us a number of answers to a very important problem. For example, does prior experience with a nonpathogenic activator have any effect on rates of complement activation *in vivo*, and what is the extent of activation of the complement system that a host develops when exposed to a specific pathogen?

Among the so-called natural antibodies, we should consider properdin. The possibility that properdin represents an antibody against a widely distributed carbohydrate cannot be ruled out at present. We can only hope that the techniques that have given us the germfree animal can be refined further to remove antigens from the diet and give us an "antigenfree" animal as well. Perhaps this will supply an answer to the question: Is properdin a truly normal antibody, that is, a part of the innate defense system of the host, or is it something that is evoked in response to an external stimulus in practically all hosts?

Let us now turn to specific antibodies, specific in the sense that we know what was responsible for their formation. In this connection some very exciting recent experiments in regard to antibody formation *in vitro* merit attention. This work, as with so many great advances over the past twenty years, was first done by Joshua Lederberg, in this case while he was with Burnet in Australia last fall,[2] and it has since been confirmed in independent studies by Monod and by Edwin S. Lennox. Essentially, the approach is very simple; a rabbit is immunized against a mixture of antigens, for example, phage antigens. The animal subsequently will produce a mixture of antibodies to the different antigens. The animal is then taken into a cold room at 0° C. and sacrificed. The lymph nodes are dissected and macerated until single lymphocytes are obtained; these are then suspended in microdroplets. To each droplet a mixture of the phages, originally used for immunization, is added. The preparation is then brought out into room temperatures, and interactions with neutralizing phage antibodies are permitted to take place. Each microdroplet is then placed on the appropriate bacterial indicator strain to see which phage has been inactivated. The results suggested that a single cell can form only one antibody; in each microdroplet only one of the several phages tended to be inactivated. I expect that by the time this monograph is published the problem of whether this means that a specific cell is capable of forming only one antibody, or whether the results may be due to interference phenomena similar to those encountered in Monod's observations on adaptive enzymes ("diauxie"),[3] will be solved. In any event, the finding that a single antibody-forming cell may be able to form only one antibody at a time might be quite important to the present discussion, and it suggests a number of interesting studies that could be carried out with germfree animals.

Quite recently Jonas Salk[4] reported that he had taken monkey heart cells, cultivated them *in vitro*, and then reinjected them into monkeys and obtained significant complement-fixing antibody titers. The monkeys were then immunized with high doses of these heart cell homogenates about six weeks later, and it was found that if the initial and the second doses were both high there was absolutely no booster effect due to the second exposure. Only when dilutions of the initial dose were made did a booster effect occur, and it increased in direct relation to the decrease in concentration of the first stimulus. In connection with the data that I have just cited on the possible formation of only one antibody by one cell, it could be suggested that Salk's observations, reminiscent of "immune paralysis," may be due to an insufficient number of antibody-forming cells capable of responding. And this brings us to the question: what could be done to stimulate the multiplication of antibody-forming cells?

Some recent data may point to the direction where a new answer to this question might be obtained. W. H. Taliaferro and B. N. Jaraslow (in press) recently demonstrated that one of the factors capable of repairing radiation damage to the antibody-forming system of rabbits is kinetin; DNA digests produced a somewhat similar result. Interestingly enough, we have succeeded in demonstrating, in our laboratory during the past two years, that a similar supplementation of certain bacterial cell systems and, according to more recent data, also of tumor cell systems (lymphosarcoma cells in the ascitic form in mice), results in a dramatic stimulation of the rate of multiplication of certain cell types.[5, 6, 7] More specifically, we found that in the case of a number of Gram-positive bacteria, particularly of pneumococci, the rate of multiplication of virulent cell types is enormously enhanced in the presence of DNA digest (DNA + DNase) and, to a more variable extent, in the presence of kinetin. Avirulent strains of the tested Gram-positive bacterial species do not respond and, as far as the Gram-negative bacterial species are concerned, those thus far tested (*Brucella abortus*, *Salmonella*) showed a response to DNA digests or kinetin involving the rather unusual selective establishment of virulent cell types in initially avirulent populations, but the actual mechanism responsible for this effect is different from the one uncovered in Gram-positive species and need not concern us here. As already mentioned, multiplication of lymphosarcoma cells *in vivo* also was stimulated by DNA digests in a manner similar to that of virulent Gram-positive bacteria, and all of these effects have proved to be independent of the actual source of the DNA employed. Quite recently W. Firshein of our group demonstrated that the stimulation of cellular multiplication under the influence of extracellular DNA digests is directly correlated with a significant increase in the intracellular content of DNA.[8] It thus would appear that the extracellular presence of an enzymatic DNA digest, or of kinetin, may be one of the requirements for the stimulation of intracellular DNA synthesis by certain cells. The aforementioned effects can be antagonized by protamines, by kinetin riboside, and by antisera against certain DNA-rich preparations which we recently succeeded in producing.[9]

One may now ask the questions: Can systems comparable to the DNA digest and kinetin systems just discussed stimulate the formation of antibody-forming cells in nonirradiated, normal animals? And, can such systems affect antibody formation and the fate of virulent types of microorganisms in germfree animals differently than in conventional animals?

Work with germfree animals might aid us considerably in the elucidation of the nature and activity of a large group of other factors that directly or indirectly affect the host's capability to cope with microbial pathogens and that may require prior activation. This would include factors that can enhance the complement system and other systems that apparently require complement for activity, such as the leukozymes and phagocytin. There is no space to discuss these here; neither is there space to discuss in any detail the interesting problem of mobilization of cellular factors that can affect the outcome of host-parasite interactions. For example, nonspecific factors capable of mobilizing leukocytes have been recognized in cases of blood cell destruction. One would like very much to know how these activations would take place in a germfree animal as compared to what occurs in the conventional animal. One would also like to know what the specificity of the systems that activate phagocytosis might be. In the latter connection we have recently made a preliminary observation which indicates that, in the case of ingestion of *Brucella abortus* cells by monocytes,[10] a DNA digest may have an enhancing effect on the rate of ingestion. This would represent a highly nonspecific factor, and one would be interested to know to what extent normally occurring breakdown products of cells in conventional animals might affect the outcome of host-contaminant interactions. Here, it would seem to me, comparative studies with the germfree animal might be most fruitful.

We have also observed, in unpublished studies conducted by W. Stinebring in our laboratory, a nonspecific interference with the intracellular multiplication of *Brucella abortus* in monocytes from guinea pigs that have been infected accidentally with other microorganisms. This is another example that demonstrates the interrelationship between prior experience of the host with a contaminant and the subsequent fate of a specific infective agent. Here again, the use of the germfree animal for a better study of such relationships seems obvious.

Finally, reference may be made to the desirability of additional investigations of a number of ecological situations that can influence the outcome of an infection, as illustrated in the case of the *Endamoeba histolytica* and *Escherichia coli* discussed elsewhere in these pages by Phillips *et al.* An additional example would be the inconsequential exposure of mice to *Vibrio cholera*, which can be changed to exposures with lethal effects following reduction of the intestinal flora by suitable antibiotic treatment.[11] The germfree animal has already been shown to be an excellent tool for this sort of study and its extended use along these lines deserves decided support.

I hope that in this rather brief discussion of factors and systems affecting host-contaminant relationships the usefulness of the germfree animal has been suggested. Since my comments must be limited by the space allotted, I have made no reference to aging and intestinal synthesis, which seem all too obvious in relationship to germfree life. I feel quite strongly that, by contributing to the better understanding of these problems, work with the germfree animal may contribute significantly to improvement of existing prophylactic and therapeutic measures against infectious agents and to the development of entirely new procedures for increasing host resistance.

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Carl C. Lindegren (*Southern Illinois University, Carbondale, Ill.*): I am a geneticist, not an immunologist, but I am concerned over the fact that, according to Braun, he obtained only a single antibody from one cell. I do not dispute the fact, but I cannot credit the inference that the cell that was sampled has been exposed to all of the antigens. This inference, I suspect, is probably incorrect because, if a single cell from a rabbit is sampled, only about one ten-billionth of the animal is sampled, and there is little reason to believe that this individual cell has been exposed to more than one antigen, except by chance.

Unidentified speaker: I was about to make this observation myself because, if I understand the matter correctly, the droplet contains 1 or 5 cells, and it might take 1000 samples exposed to 1 type of phage and another 1000 to another type of phage to make sure that no 2 types of antibody can be formed by 1 cell. Unless I am mistaken, the statement has been made that there is some evidence that 1 cell has formed antibodies against 2 types of phage. In these experiments positive evidence is of much more significance than negative evidence.

I shall also comment on the possibility that germfree animals may have some latent viruses. Probably everyone remembers the history of the testing of the Salk polio vaccine. Ten laboratories tested it in monkeys and in tissue culture, but only 2 had the foresight to use cortisone in their animals, with the result that these 2 laboratories could detect poliovirus in their monkey tissues while the other 8 could not. It might be possible that such hidden viruses might show in germfree animals treated with cortisone and other such agents. While I do not feel that further comment is needed, I should like to say that the examples that I gave were merely reports and were intended only as examples to illustrate my points. I am confident that the question as to whether one cell forms one antibody will be answered in the very near future.

Floyd Daft (*National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md.*): The techniques that have been described in this monograph certainly should be of very great assistance in the solution of many of the problems of metabolism in metabolic diseases. This should be true not only of the germfree technique itself; there is also the equal and perhaps even greater possibility of using a controlled bacterial environment by contaminating the germfree animal with one or more pure cultures.

Some of the problems that seem to lend themselves to the germfree technique are: dietary liver necrosis, experimental liver cirrhosis, and adrenal necrosis found in pantothenic acid deficiency. These are examples of problems in which the germfree animal should be of critical importance in determining the precise etiology of the disease in question.

Paul György and his associates at Western Reserve University, Cleveland, hio, have presented, from time to time, evidence in support of the concept hat dietary liver necrosis in rats may result from the impact of toxins produced y bacteria of the gastrointestinal tract on the liver weakened by dietary inult.[1, 2] The work of Fine and his associates is interpreted as indicating that xperimental liver necrosis associated with choline deficiency is probably a irect result of bacterial invasion. Their data seem quite convincing.[3] I beieve their theory has not yet been tested with germfree rats, and it would seem hat such an experiment could prove or disprove this concept.

Hemorrhage and necrosis of the adrenals, which occur with some frequency n pantothenic acid-deficient rats, might also conceivably result from bacterial ction, although I know of no evidence to date that suggests this. However, e have made a few studies on pantothenic-acid deficiency in germfree rats, nd as yet we have seen no adrenal necrosis in these animals.

These few examples indicate the general point that in many metabolic deects or abnormalities it is quite within the realm of possibility that there is a acterial toxin or a viral component and, to settle such questions, it seems bvious that perhaps the only direct tools at our disposal are the germfree nimal and the techniques discussed in this monograph. It might be presumpive to classify aging as a metabolic disease, but I think that few would deny he relationship.

To those of us who have available the instrumentation for the study of germree life and animals with a controlled contamination, such problems present a ormidable but stimulating challenge. In many problems, it would seem, the ermfree animal is insufficient, and investigation of it must be coupled with the ontrol, rather than the total elimination, of the bacterial flora.

Our own studies at the National Institute of Arthritis and Metabolic Diseases have so far dealt with relatively simple problems related to the possible eneficial effect of the intestinal bacteria. More specifically, they have dealt ith the synthesis of vitamins by intestinal bacteria and the availability of such ynthesized vitamins to the host. The studies thus far attempted have dealt nore specifically with the question of the need of exogenous folic acid by the at. In another group of these studies we are attempting to elucidate the nechanism of the prevention of vitamin-B deficiency by adding a large amount f vitamin C to the diet.

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H. A. Gordon (*Lobund Institute, University of Notre Dame, Notre Dame, Ind.*): n reference to the question raised by Daft "Is aging a metabolic disease?" shall mention a structural aspect of the liver that is possibly associated with he "physiological" effects of the flora.

It has been shown in a wide variety of animals that the diameter of the liver lobule is gradually increasing from birth to senescence. This process is assumed to proceed more or less parallel to the lengthening of the liver capillary and with the increase in the mass of the liver that takes place in the course of conventional life.[1] The substantial deficit in the relative weight of the liver that we have been able to observe in some germfree species has caused us to speculate on the role of the intestinal flora in this "physiological" change of the liver.

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Reyniers: Apropos of this discussion the question has been raised as to the cause of death in germfree animals. Concomitant with this has been the implication of aging.

There is no definitive answer to these questions simply because germfree animals are seldom held to the ends of their natural lives. They are too useful for other experiments, and the needed equipment has not been available for such long-range studies. However, in the course of a cancer research program it has been necessary to hold rats and mice for extended periods. Since they were selected for tumor formation, such animals are not the animals of choice for a study on aging, nevertheless we have made some observations on the C3H mouse that may be of interest.

In the C3H germfree mouse observed through 7 generations and for periods up to 900 days or even longer, fed on a diet that permits reproduction and growth, the males by far outlive the females. The females, when held for extended periods, have all succumbed to metastatic calcification. This does not occur as grossly in the germfree male mouse or in either male or female conventional C3H mice on the same diet. Tumor formation in the germfree C3H mouse is very low or absent if the animals are well nourished, whereas 90 per cent of the conventional females of this strain succumb to mammary tumors around the tenth or eleventh month, and very nearly 99 per cent do so by the nineteenth or twentieth month. In the conventional males the incidence of hepatoma is about 40 to 50 per cent between the fifteenth and sixteenth months of age. As far as longevity in the germfree males is concerned, some are more than 900 days of age at this writing. My impression is that in old animals the urinary system is the first to show outstanding breakdown. However, this must remain a mere observation at present.

The germfree white Swiss mouse, which was also used in these studies, does not exhibit marked calcification in either males or females. The germfree white rat over 1000 days of age does not show such calcification, but both the Swiss mouse and the white rat show renal degeneration at an advanced age.

In order to make the utmost use of these animals held for long periods we have packaged their tissues and products and are distributing them among investigators who are interested in aging. What their studies will show is difficult to tell. I hope that this has added a mite to the subject, but it must be understood that until quite recently those of us engaged in germfree research had neither the equipment nor the time to undertake extended studies in this area. That this should be done under proper conditions of control and planning is, of course, obvious.

WAGNER: May I add a small item to Gordon's observations on liver aging. I think it is proper to assume that the liver in germfree animals is relieved of some of its functional duties, particularly those involved in the detoxification of absorbed intestinal products of bacterial origin. In conventional rats and mice, indole is produced and readily absorbed from the intestinal tract. It is detoxified by the liver to indican and excreted in the urine. We have recently reported that the germfree animal does not show fecal indole or urinary indican. This is one indication that the germfree animal liver is relieved of some of its work.

LUCKEY: I should like to offer a thought that might relate to the remarks of Reyniers and Wagner and that should anticipate the question of the standardization of diet that always arises when matters of this kind are considered.

With respect to the immunologist, may I ask: "Just what do you mean by an antigen-free diet?" Is this really what is wanted? Shall we begin with gelatin, sucrose, corn oil, synthetic salts, and vitamins? If such a diet were defined, it might be produced.

This leads to the consideration of another aspect of germfree life implied by Daft, and I should like to point out further that the germfree animal is very useful in studying diet or nutrition per se. I am thinking primarily in terms of defining nutrition, which cannot be done in a polycontaminated rat, because as soon as we have fed the animal we have also fed bacteria. At this point no one can know exactly what is occurring from the nutritional standpoint. Only the germfree animal has the potentiality of permitting us to study nutritional physiology and allowing a critical definition of nutritional principles. To a nutritionist standardization means a synthetic diet, a diet for which a formula can be written. If this can be done and the germfree animal is used to test it, the principles of nutrition can be brought to an exact basis.

DAFT: I appreciate the comments of the various investigators. I believe that one of the problems in which everyone should be interested is the use of the germfree animal or one with a controlled flora in studies concerning aging and longevity. As Reyniers has said, the only reason that none of us have begun such studies thus far is that they tie up apparatus for long periods.

WRIGHT: Throughout this monograph we have encountered differences in terminology. We shall now consider the need for an adequate terminology for studies concerned with germfree life. Theodor Just will introduce the topic.

THEODOR JUST (*Chicago Natural History Museum, Chicago, Ill.*): The historical aspects of this subject were beautifully reviewed by Reyniers and his associates almost ten years ago in Lobund Report No. 2.[1] I can begin immediately, therefore, with the critical discussion of where we go from there.

Terminology may be considered from two points of view: first, semantics and, second, conceptual background. In other words, what is the framework to which the multitude of observations and techniques can be attached and what is a common denominator that will be useful after all this is assembled?

I call your attention to a similar development of recent years in the field of evolution. Ever since this subject has been considered from an ecological point of view based on the species concept, tremendous strides have been made. Because of a similarity of situations, it would be well to approach the subject of an adequate terminology for germfree life studies from an ecological basis.

What impresses me most of all in these discussions and descriptive accounts of germfree life and the techniques needed to produce it is that we are working with a well-defined environment or a closed environment. This, I think, is a feature to be stressed.

I should like to compare this also with the various controlled environmental experiments being done by botanists and others who concern themselves primarily with control of the physical environment, whereas in germfree life studies physical environment is incidental, and the ultimate goal is the germfree host. This special environment should be so designated by a series of terms. We could speak of a microecological environment or of a stenoecological environment. There are terms, then, such as these that could be used as a means of designating the particular technique, as well as the animal itself.

The late Raymond Pearl postulated four criteria for the description of the habitat.[2] The relationship of an organism with its environment may be classified as follows: first, the relationship is specific for every particular species or organism; second, it is continuous, that is, the organism lives in its environment for its entire life; third, the relationship is reciprocal to the extent that the environment affects the organism and vice versa; and, fourth (I am sure this has been challenged by the work done in germfree life), these relationships are indissoluble, meaning that the organism could or should not be dissociated from its so-called conventional environment.

Ecologists usually refer to the interactions between various members of one and the same species as coactions. For the sake of the record I mention the term allelopathy proposed by the botanist Molisch in 1937.[3] This term describes these coactions, which may extend between members of one and the same species or between one species and another in communities.

The term symbiosis is widely used. However, it has become customary to restrict it to the invertebrates and to plants. Recently a group of five Chicago ecologists have redefined this term by returning to its broad original definition, which included all phases from parasitism and commensalism to mutualism and, in a sense, the animals that, on the whole, are broadly symbiotic with the plant kingdom.[4] This is certainly a broad term with which to work. The term symbiosis is important to our discussion because it brings us to the level of the individual, and I am sure that this is the level at which the terminology of germfree investigation will eventually be resolved. We have heard that the germfree state is a relative state; it is an intraorganismal and, more specifically, a cellular state.

On the whole, it seems that symbiosis in vertebrates is far more uniform than it is among lower animals and plants. In a very interesting summary of the interaction of microorganisms, Burkholder gave a really elegant scale of 9 systems of coaction that may be detected in all microorganisms.[5] Unfortunately, space does not permit consideration of them here.

Buchner, in his classic monograph on endosymbiosis in lower animals,[6] has given us a term that I think is of interest because the entire field of symbiontology is largely a matter of describing the mechanisms, devices, and locations of the microbiota in the invertebrates.

I have made previous reference to controlled environmental experiments pertaining primarily to the physical environment, the phytotron, and we could perhaps construct a corresponding zootron or biotron. Insofar as our needs here are concerned, I believe that we should seek a term that is clear, simple and flexible.

I think one significant point in regard to the germfree animal is the fact that it is left to its own resources. We might call this a condition of known life. We might describe it by translating the term germfree into Greek as amicrobiotic or some similar term comparable to this. If we take a positive approach and say that the germfree animal is on its own we could speak of an "autobiont or an idiobiontic organism," which is entirely on its own resources.

In desperation I went to a dictionary,[7] and the best that I could find in it was the term bion, which is short and implies a morphologically independant organism. It is close enough to the term *bios*—life—to be familiar. I therefore suggest the word biontology, which would cover the entire field, both plant and animal. and would imply an independent organism. We could proceed further and develop biontological formulas that would indicate, in codelike or shorthandlike fashion, the criteria that are set for any particular experiment and thereby transmit this information conveniently. Such formulas are in use in various phases of biology. One could expand further and speak of an organism that contains a single contaminant as a monobiont, one with two contaminants as a dibiont, and so on. I call your attention still further to the fact that in botany we use similar terms such as haploid and diploid which, however, have reference only to the chromosomal constitution of the organism.

Therefore in closing, I recommend to you the term biontology to describe the field of germfree life.

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Question: May I ask whether the term biontology would not be confusing? If the present word ending were replaced, would this not obviate confusion?

Just: We have the term bion, which eliminates the suffix, but this gives an entirely different concept of unity in ecology. I believe that foreshortened forms of some of these terms, such as biontology for symbiontology, will probably be the terms used. If we can drop prefixes or suffixes, by all means let us do so; we have already taken so many liberties with Greek grammar that we can afford to take a few more.

T. D. Luckey: Perhaps we could subsitute the letter *t* for the *n* in some places. This has been done on several occasions, and it makes a very fair and decent system to refer to a monobiotic rat or monobiot. In this manner a dibiotic animal would be one with a single contaminant. We could also refer to abiotized or monobiotized animals. Would this be acceptable?

Just: There is the objection that the term biota is already in wide use for the sum total of ecological productivity of a given region, so the assigning of a second meaning to the word would probably cause additional confusion rather than simplification. I regret that so many of the terms suggested are already in use in other contexts, but this is one of the difficulties inherent in word coinage.

Luckey: While the word biota could not be really useful to us, perhaps the word biotology might.

Gordon: An added problem in this field is a proper name for the ordinary "normal" animal. In gnotobiotic experimentation some of us refrain from using the word normal in its usual sense because it carries the connotation that the gnotobiotic animal is not a normal animal. This may be true by ordinary standards, but if our definition of normality refers to the adequate state of adaptation to environmental conditions, which some germfree species do display, then we have reason to call the germfree animal, or indeed any gnotobiotic animal, a normal one. Furthermore, the expressions normal or normal control are also needed in gnotobiotic experimentation for contrasting the "experimental" animal of a given series. Thus, in the past the expressions conventional and normal stock have been used to denote the ordinary polycontaminated animal. None of these expressions appears to be completely satisfactory in its meaning, yet the selection of and agreement upon a common term is distinctly needed.

Frank J. Orland (*University of Chicago, Chicago, Ill.*): The difficulty that we have encountered in this matter of terminology appears to be the result of the vast variety of concepts that are held at present. We have had little of such difficulty in this monograph because we all understand each other quite well, being able to define concepts as they have arisen. Moreover, this is because we have been using laboratory parlance, and we have defined various situations as we encountered them. For the future, and especially in publications, it is very important that we agree upon terminology and formulas so that we shall know what we are talking and writing about as the field of germfree research expands.

Most certainly, in our own experience and in our correspondence with the Lobund Institute group, we have encountered the need for a definitive terminology. We have evolved a formula of numbers and letters that can designate a given germfree animal, on a given diet, and inoculated with a given microorganism. However, this has been merely a matter of convenience between our two groups. However, as the editor of a research journal, I feel that we must look far beyond such expediency. How will poorly defined terms look in the literature? How foolish will they appear after several decades? It is imperative that we have a terminology upon which there is fairly universal agreement at this juncture. Such a term as "gnotobiotics" will have a good deal of usefulness; indeed, this term for the total field of study has already been established in the literature.

E. L. R. STOKSTAD (*Lederle Laboratories, Pearl River, N. Y.*): This discussion reminds me of our experience with folic acid. This term was introduced about ten years ago. After we had determined its structure we gave it a very good chemical name, pteroylglutamic acid, that described it accurately and permitted the naming of other analogues; however, very few used the term. Irrespective of whatever term we may select here, there will be investigators who will prefer the term germfree.

JUST: Please remember that I have not discarded the terms germfree or gnotobiotic. Speaking as an editor, may I point out that the Government Printing Office, Washington, D. C., has long ago done away with some terms, even though the biological literature is still filled with them. We frequently find such descriptive terms hyphenated, and there is one distinguished botanical friend of mine who asked, "Have you ever met a botanist who could pronounce all these hyphens?"

CAVETT O. PRICKETT (*E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.*): My topic, "The Knowledge of the Development, Anatomy, Biological and Physiological Characteristics of Germfree Animals Requisite Before Their Use in Experimental Research" is so broad in scope that I suspect it was designed to permit latitude in questions and discussion. All I can do is to reiterate and summarize some of the anatomical, physiological, and biochemical differences that are known to occur in germfree animals as compared with animals reared in conventional environments. In addition, I shall attempt to point out some of the problems one may encounter in promoting the use of germfree animals or the germfree technique for the elucidation of biological phenomena.

The germfree animal has been defined by Reyniers as one that is "free from all demonstrable living contaminants." Thus, the technique permits the standardization of laboratory animals for experimental purposes and/or the standardization of the procedures used. It has been compared with the pure-culture concept exemplified by, but by no means confined to, microbiology. The conventional animal is a mixture of the animal and its microbial flora living in harmony. As is pointed out elsewhere in this publication, the elimination of the flora represents only one step in the development of absolute standardization, since the germfree animal may not necessarily be living in an environment where sufficiently definite and/or desirable parameters may be discerned. In comparison with animals reared in the germfree environment, conventional animals show great variation in degree of standardization, since the conditions under which animals are reared, cared for, and used for experimental purposes differ considerably in various laboratories. These variations often are reflected in changes in the anatomy and physiology of the host. Gordon describes in this publication the effect of feeding antibiotics to conventional animals and the resultant decrease in lymphoid tissues. The relative importance of such changes can be measured only by determining their relationships to the definitive answers required by the particular type of experimental design.

Biological variation is reflective of intrinsic variables, such as genetics, physiology, anatomy, and psychology, that are a part of the make-up of the animal itself. While standardization of intrinsic variables is possible, the subject under consideration refers most pertinently to standardization of the animal and its extrinsic or physical environment. In the case of the germfree animal, this means to divest him of his bacterial flora and thus eliminate it as a variable.

As the Lobund Institute group has reported previously, germfree animals show a number of differences when compared to conventionally reared animals.[1, 3-7] The intestinal wall is shorter and thinner, the cecum is characteristically larger, with fewer lymphoid areas and a decreased blood supply. There also appears to be a lessened amount of areolar tissue and a flaccid musculature. The liver is usually smaller. The germfree animal is said to mature faster than its conventional counterpart. No antibodies are present, but defense mechanisms can be activated with appropriate antigens. At death, there is normal enzymatic activity, but no putrefaction, since the animal contains no bacteria.

One interesting recent finding by Reyniers is that the germfree female mouse of the C3H strain shows extensive calcification of the kidney and sometimes of the heart and lungs. This metastatic calcification is not seen in the germfree male or in conventional males and females on the same diet. In addition to the above, the work at the Lobund Institute has demonstrated certain other differences, such as the reaction of the germfree animal to microbial contamination and the lessened susceptibility of the germfree host to X rays.

While all these changes may be due in large part to the germfree individual's lack of experience with microbial flora, one should not ignore the fact that this animal also exists in a very refined environment as compared to its more conventionally reared counterpart. Thus, in assessing the differences apparent between the germfree and the conventional animal, we should assure ourselves of the importance of each extrinsic and intrinsic factor involved.

During the last fifteen years I have observed many phases of the biological studies at the Lobund Institute, as well as a few cases in which the technique was useful in the successful culmination of chemical and physical problems.

The extrinsic variables, such as housing, noise level, diet, temperature, humidity, physical activity, particle size and biological availability of nutrients, all contribute to differences that as yet cannot be entirely attributed to the lack of microorganisms. As compared with the intrinsic variables (genetic, physiological, anatomical and psychological), the influence of the extrinsic variables are relatively easy to assess and define.

The amount of lymphoid tissues seen in the germfree animal is described as being "fetal" in type. Reduction in the amount of lymphoid tissue can also be produced by variations of environment in conventional animals. For instance, the chicken, which has no well-defined lymphatic system as has the mammal, but has isolated foci of lymphoid tissue throughout its body, shows a considerable difference in lymphoid tissue development in commercial poultry as compared with experimental chickens. In chickens, foci of lymphoid cells are also subject to genetic variation. The mammal, having a formed lymphatic system, is more difficult to assess; however, it is apparent that the lymphoid tissue seen in germfree mammals is less developed as compared with conventional animals. Development in lymphoid tissues is also subject to diet and to the influence of hormones and, in germfree life, may reflect lessened, or perhaps greater (?), stresses imposed by the germfree environment.

Finally, variation in lymphoid tissue in the chicken is often influenced by both the structure and content of the diet. Those who have worked with animals on synthetic diets know that, in the case of the chick, the crop and the gizzard are poorly developed and, in the case of the bovine, the use of synthetic or semisynthetic diets from birth reduces the size of the stomach.

Decreased muscle tone may be a reflection of the physical nature of components of the diet, and it is also probable that the germfree animal exists in an environment where it has lessened physical activity.

In the assessment of the anatomy or physiology of the germfree animal there is little to indicate the presence of any actual pathology, except for the recent finding of Reyniers with respect to metastatic calcification in the C3H female mouse, but not in the C3H male. This may be of dietary origin and may reflect a lessened need for the usual calcium supply and/or a mineral imbalance. Changes in organ size or weight are of limited importance unless dysfunction is proved.

In the field of chemotherapy, the particle size of a candidate chemical often markedly influences its efficacy and pharmacology. This is a very difficult factor to control, but I can recall a situation in which, on the first test of a chemical, the material was efficacious and nontoxic at one-half per cent in the diet and, after refinement, it proved too toxic for practical use at a level of 0.0027 per cent of the diet. The only difference in this material was the smaller, more uniform particle size. Incidentally, efficacy and toxicity showed little parallelism. From a nutritional standpoint, the physical form, the biological availability of ingredients, the proper adjustments in requirement levels for a relatively uninfluenced host, as well as the lack of competition with microbial flora, may account for some of the differences seen. There are many cases in which the germfree animal would offer ideal material for the elucidation of nutritional problems. For instance, a recent report by Morgan and Yudkin[2] suggests that sorbitol can substitute completely for thiamine and other B vitamins in the diet of rats. Under these circumstances, a normal growth curve is exhibited and no deleterious effects are seen. The authors attribute this substitution, in part, to the possibility of the flora entering into the picture, with resulting synthesis of the required B vitamins. Other areas such as growth-promotion by antibiotics and the known dependence of antibody response on the nutrition of the host offer potent opportunities for expression through the use of germfree animals or the germfree technique.

In summary, the germ-free animal represents, to a greater degree than was previously available, a standardized norm. However, comparisons between the germfree animal and conventional animal may, in some cases, have no justification. Biological sequences exist in certain areas of experimentation involving pathology that may have no counterpart in the normal. They are not necessarily either an exaggeration or a diminution of any normal process. In such cases the definition of a normal picture would be of limited usefulness.

The definition of many of these problems represents a long-range effort and it should not be assumed that their completion is necessary to utilization of the germfree animal and/or the germfree technique for studies which are of great immediate importance. The requisite experimental design, in each case, will determine and dictate the level of necessity for basic background data in order to obtain definite and meaningful answers.

There is great truth and great merit in the statement often made that "even in the most purely logical realms, it is insight that first arrives at what is new."

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LUCKEY: The subject of standardization has been raised many times, and I feel that, even though the germfree animal has not been completely standardized, we should remember what has been accomplished in this respect.

In regard to the particle size of the diet I agree with Prickett, and I think all of us have been lax in respect to the physical state of the diets used. I am also sure, as he states, that in the future people will define such data as the size of the cages in which germfree rats were held, how much water and food they used, whether feeding was on an ad libitum basis and, if so, why, what the noise level was, how much dust was in the germfree isolator, psychological factors, and association with other animals in the same isolator. As a matter of fact, if a rat is petted 5 minutes a day there may be a 10 per cent increase in its growth rate. Smell, taste, and feel are also important factors. We keep records of temperature, humidity, and light, but we do not always report these or attempt to standardize them from experiment to experiment. In spite of the fact that it is possible to control a germfree environment we do not always do so, and I think this is a responsibility for the future. The fact that this need for control is beginning to appear in discussions and in publications is encouraging. We do well enough bacteriologically and, to some extent, in specifying the diet, but I do not think the diet can ever be standardized unless it is completely synthetic and a formula can be written for it. This may not happen very soon.

The other point that Prickett mentioned is genetic control. We do not have this to any great extent, and we should be conscious of the need.

In passing, I propose a new laboratory animal, the small armadillo from South America. These animals produce about 95 per cent of their litters as identical sibs. Among other reasons for their use is their size. Apart from the possibility of using these animals in germfree research, they should be examined for suitability in conventional work. The point simply is that the need for control of environmental and other variables is now before us and should be taken seriously.

PRICKETT: There are studies in the literature on the influence of noise levels, and it is believed to have profound effects on the growth of rats. Perhaps the germfree animal is not subjected to the same noise levels as the conventional animal, and this could account for differences in rate of growth.

ANTONIO ROTTINO (*St. Vincent's Hospital, New York, N. Y.*): I shall discuss briefly the germfree technique and its applicability to the establishment of the etiological significance of microorganisms isolated from diseases of unknown cause. I shall also discuss a consideration that must be weighed before reaching a final conclusion as to why tumors fail to develop spontaneously within the normal time range when this technique is used.

Our work on the etiology of Hodgkin's disease[1] is an illustration of the first point. For more than 75 years numerous workers have sought a causal agent for this baffling disease, and a large variety of organisms have been suggested at various times. One by one, each has been shown to be either a contaminant or a secondary invader. One bacterium was especially difficult to eliminate as being of no significance: namely, a pleomorphic acid-fast diphtheroid first described in 1914 by Bunting and Yates[2] as the bacillus of Hodgkin's disease. In 1916 Cunningham[3] believed that he had shown it to be a contaminant, and there the matter rested until 38 years later, when Fleisher[4] resurrected the organism at about the same time that we were also isolating it from the blood of our Hodgkin's disease patients. TABLE 1 summarizes our experience.

In our first experiment the blood of patients with Hodgkin's disease was cultured in ordinary tryptophan broth, and 20 per cent of the cultures were positive. Repetition of the experiment on blood from the same group of patients produced only 10 per cent positive cultures. We then changed the culture medium to the pleuropneumonialike (PPLO) medium and, much to our amazement, we obtained 92 per cent positive cultures. We then repeated the experiment on a large number of blood specimens, this time culturing in a small sterile room with closed door and still air. Under these conditions, 44 per cent of the cultures were positive. The experiment was again repeated

TABLE 1

Culture Conditions and Results

	No. of cultures	No. positive for diphtheroids	Per cent positive
(1) Open laboratory, tryptose broth	55	11	20
(2) Open laboratory, tryptose broth	53	5	10
(3) Open laboratory, PPLO medium	26	24	92
(4) Sterile room, PPLO medium	137	61	44
(5) Sterile room, PPLO medium, blood drawn in operating room	48	4	8
(6) Germfree hood, PPLO medium, blood drawn in operating room	60	0	0
(7) Sterile room, PPLO medium, blood from tumor-bearing mice	13	4	30
(8) Germfree hood, blood from tumor-bearing mice	8	0	0

with still greater care in taking the blood samples. All blood was drawn in the surgery, using sterile surgical technique and containers, and the culturing was done in the sterile room. Positive cultures fell to 8 per cent.

At this point we sought the help of Reyniers and, in accordance with his suggestions, constructed a simple box or isolator that could be operated germfree. The results were clean-cut. Fifty blood specimens were cultured, and all were negative. It took two years to complete this work.

I think this illustrates the fact that even the most stringent asepsis is not sufficient when dealing with microscopic organisms, and that asepsis must be supplemented by the germfree technique to obtain unequivocally dependable results.

In regard to the work of Reyniers, the question arises as to the role environment plays in the failure of tumors to develop in germfree animals. I use the term environment in the broad sense, as including the germfree state. In experiments with *Drosophila* tumors it is an established fact that variations of temperature in which the flies are reared profoundly influence the incidence of the tumor. In mice, Tannenbaum[5] has shown that diet influences the tumor rate and incidence. Could it be that the germfree environment so influences the physiology of mice that the tumor factor, if present, is unable to express itself under these conditions? I believe this question must be answered before failure of tumors to appear can be ascribed to absence of any tumor-inducing factor.

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Reyniers: Elsewhere in this monograph I reported on cancer experiments using germfree chickens and the subsequent isolation of biologically active subcellular particulates, which have been further purified and pictured by Taylor. These particulates are from methylcholanthrene/benzene induced tumors in germfree chickens. I did not report on our work with germfree mammals, and I can only briefly sketch the outline of these studies.

In C3H mice (Tannenbaum substrain), 90 per cent of conventionally reared females are dead of mammary carcinoma between the tenth and eleventh months and from 98 to 99 per cent are dead by the eighteenth to twentieth months. The males develop hepatomas; that is, 40 to 50 per cent by the fourteenth to sixteenth months. The genetic control used in conventional stock was continued in the germfree animals by brother-to-sister matings. The diets used were those employed in rearing germfree C3H mice, and the genetic pattern was duplicated. It is too early to report definitive findings, but this much may be said: in a group of more than 100 germfree C3H mice reared through 7 generations, the oldest animals being more than 700 days from the first to third generations, no mammary tumors and no hepatomas were observed. I caution against the conclusion that tumors do not appear in germfree C3H mice; at this point we do not know what stress or deliberate injection of carcinogenic substances will do. We do not know what will happen in repeat experiments and in larger groups of these animals. We are prepared to say at present only that tumor formation is delayed, and that tumors that are characteristic of this strain of mice have not appeared within the limits of the observations. Whether this is due to environment, the state of being germfree, or because by rearing germfree C3H mice we have eliminated the Bittner factor we are not prepared to say now.

Tumors can be caused in germfree C3H mice by injecting a carcinogen, and such tumors may be transmitted from germfree mouse to germfree mouse by tumor homogenates. Apparently they can also be transmitted spontaneously (perhaps this is not the term to use), from mothers in which a carcinogenically caused tumor has been transmitted to the offspring in which tumors appear spontaneously.

It is of further interest to report that germfree C3H mice taken from an isolator and conventionally contaminated and bred into a colony in the same room with conventional C3H mice, but not bred to them, also show the same general low incidence or, if you will, absence of tumors within the abovementioned period.

I mention the work with Swiss mice and white rats only to say that, in general, the first observations are the same as with C3H mice. However, I must emphasize the fact that we are not interested in eliminating cancer from these mice, for this is not the design of our experiment. We should prefer to be able to obtain a germfree strain of animals in which spontaneous cancers appear, and it is to this end that we are working. If this occurs, then it may be presumed that whatever agent is responsible for such tumors, if it is an agent, will be trapped in a system in which no other demonstrable microbes exist. The identification of such an agent should be much simpler than in a conventional animal. We believe we have this condition in one of the sublines of germfree white rats and in Swiss mice.

I hope this will partially answer Rottino's question, and I regret that neither space nor information is available to do so completely. I do not desire to leave the impression that either my associates or myself claim a virus in the cancer phenomenon as we see it in germfree mice or rats. We prefer not to involve ourselves in this rather sterile argument at this time.

It is now thirty years since I began work on germfree life. It was a lonely path at first, not without ridicule and pain but, like all these journeys, the way has been made pleasant by many friends and collaborators, as well as by my staff. Arriving at the situation today, I am about to start on a new road still marked by germfree life, but in a new direction and in a new location.

In attempting to summarize the things that this monograph has brought out it seems to me that one thing is evident. The long period of emphasis on techniques and instrumentation is past. The germfree animal no longer causes wonderment among scientists or even in the press, and this is as it should be. Adequate techniques for rearing animals and conducting experiments in a germfree environment are available. Improvements may be expected to take place in these techniques, but the emphasis must now pass to the study and use of the animals.

One thought more: I hope that for the future none of us working in this field will fail to recognize the responsibility that is ours to study the germfree animal itself in spite of all the attractiveness and glitter of the practical problems it can solve. Premature—and I am tempted to say irresponsible—use of the germfree animal before the animal is itself known and before careful consideration is given to the control implied in the technique, could be detrimental to the future development of this field. The germfree technique and animal are still relatively new, and we all have the opportunity to build solidly. To me this means planned, carefully coordinated work between individuals and centers. Certainly, this has been the effort up to the present, and I can assure you that such a systematic program laid out at the University of Notre Dame in 1930 has achieved precisely what it started out to do and has not deviated. This reflects no great credit to me; whatever credit there is belongs to my associates and to the University of Notre Dame.

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